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Enhancing non-viral cell transfection through lysosomal escape mediated by listeriolysin O

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There's not a problem that I can't fix, ´cause I can do it in the mix!

Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Micro liter
µs	Microseconds
4IPBA	4-iodophenylboronic acid
A	Absorbance
AA	Acryl amide
aa	Amino acid
Ab	Antibody
AlPcS2a	Aluminum phthalocyanine
as	Antisense
BASO	Basophils
Bis	N,N-Methylenebisacrylamide
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BPB	Bromphenolblue
BSA	Bovine serum albumin
C	Carboxy
cDNA	complementary deoxyribonucleic acid
CMV	Cytomegalovirus
d	Day
D	Dalton
dd	double distilled
DMEM	Dulbecco´s Modified Eagle Medium
DMRIE	N-[1-(2,3-dimyristyloxy) propyl]-N,N-dimethyl-N-(2-hydroxyethyl)ammonium bromide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	2´-deoxynucleoside-5´-triphosphate
DODAC	dioleoyldimethylammonium chloride
DODMA	(*2,3-dioleyloxy*)propyl)-N,N-dimethylammonium chloride
DOPE	dioleoyl phosphatidylethanolamine
DOSPA	*2,3-dioleyloxy*-N-[*2*(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
DOTMA	N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-trimethylammonium chloride
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EOS	Eosinophils
EtBr	Ethidium bromide
EYFP	Enhanced yellow fluorescence protein
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
G	Guanine
GAD65	Glutamic acid decarboxylase 65

GAP	GTPase activating protein
GFP	Green fluorescence protein
GH	Growth hormone
Gnd-HCl	Guanidine Hydrochloride
h	Hour
HA	Hemagglutinin
His	Histidine
HRP	Horse radish peroxidase
Hz	Hertz
IONP	Iron oxide nanoparticles
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kb	Kilo base pairs
kD	Kilo Dalton
L. monocytogenes	*Listeria monocytogenes*
L2K	Lipofectamine 2000
LB	Luria Broth
LLO	Listeriolysin O
LYM	Lymphocytes
M	Molar
MCS	Multiple cloning site
mg	Milligram
MHz	Megahertz
ml	Milliliter
mM	Milli molar
MOMP	Major outer membrane protein
MONO	Monocytes
MPa	Megapascal
mRNA	Messenger ribonucleic acid
N	Amino
n	Number of experimental repeats
NC	Nitrocellulose
NEU	Neutrophils
ng	Nanogram
Ni-NTA	Nickel nitrilotriacetic acid
NLS	Nuclear localization signal
nm	Nanometer
NPC	Nuclear pore complex
OD	Optical density
ODN	Oligodeoxynucleotides
P	Pellet
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylene imine
pg	Pico gram
pH	Potentia Hydrogenii
PLGA	Poly(lactic-co-glycolic acid)
PLL	Poly-L-lysine
pmol	Pico mol

PrV	Pseudorabies virus
RBC	Red blood cell
rpm	Rounds per minute
RT	Room temperature
s	Sense
SDS	Sodium dodecyl sulfate
sec	Second
SiO2	Silica
SN	Supernatant
SV40	Simian virus 40
T	Thymine
T1D	Type 1 diabetes
Taq	*Thermus aquaticus*
TBE	Tris boric acid EDTA
TEMED	N,N,N´,N´-Tetramethylethylenediamine
TfR	Transferrin receptor
Tris	2-Amino-2-(hydroxymethyl)-propan-1-3-diole
U	Unit
V	Volt
VEE	Venezuelan Equine Encephalitis
VEGF	Vascular endothelial growth factor

Contents

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1. Introduction

In contrast to strategies based on the introduction of transgenic cells expressing growth factors (*ex vivo* therapy), or the direct administration of recombinant growth factors into target systems, *in vivo* gene therapy approaches (introduction of therapeutic plasmids encoded for growth factors) provide a promising alternative associated with lower manufacturing costs, higher safety and increased bioactivity of the produced proteins (due to host-specific post-translational modifications and correct folding of the locally produced growth factors). In order to introduce exogenous DNA into cells *in vitro* and *in vivo*, several strategies based on viral or non-viral approaches can be applied.

Viral gene transfer strategies (retroviruses [1, 2], adenoviruses [3, 4], adeno-associated viruses [4-7], herpes viruses [8], lentiviruses [9] and epstein-barr viruses [10, 11]) show by far the highest transfection efficiencies *in vitro* and *in vivo*, whereas non-viral vectors (polycations, cationic lipids (section 1.1.2 and section 1.1.3), and mechanical methods (1.1.1)) are limited in their efficacy to deliver genes, especially in the presence of serum and other proteins, which makes unmodified non-viral vectors inapplicably for *in vivo* approaches. But nevertheless, due to safety aspects, the application of non-viral gene delivery systems *in vivo* gain more popularity due to the high disadvantageous potency of viral systems to promote detrimental immune responses. Additionally, the viral tendency to integrate introduced exogenous DNA into the host genome dramatically increases the probability of tumor cell growth in target cells. Furthermore, non-viral vectors score concerning their manufacturing cost and easy handling during gene therapy approaches.

To conclude, it is more worthwhile, easier and non-risky to increase the efficacy of the gene delivery using non-viral vectors by specific modifications or additional auxiliary substances, than trying to make viral systems safer.

The following sections give an overview about physical and chemical barriers hindering the delivery of macromolecules into eukaryotic target cells, and the corresponding solutions for overcoming these delivery limiting obstacles. An approximate overview about possibilities to gain access into the target cell is depicted in Figure 1.

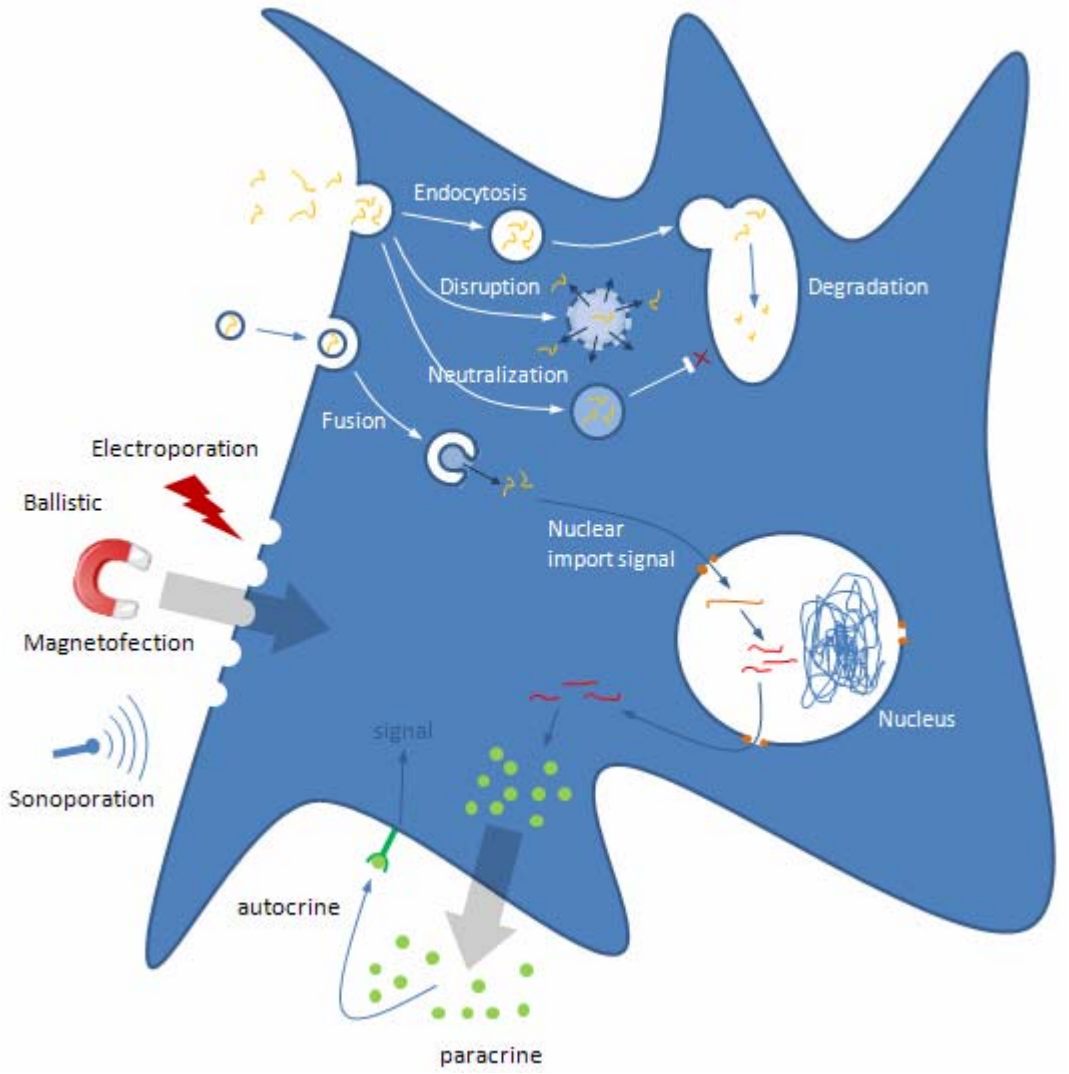


Figure 1: Quick overview about barriers and solutions for an efficient delivery of DNA and other macromolecules

1.1. Overcoming the cell membrane

Although the transfection efficiency and the level of gene expression is dependent on various factors such plasmid size and copy number [12], the transfection efficiency in general using nude DNA (DNA without auxiliary carrier systems, section 1.1.2), showed weak efficacy, independently of the targeted cell and tissue type *in vitro* and *in vivo,* respectively.

1.1.1. Mechanical/physical methods

1.1.1.1. Magnetofection

Magnetic nanoparticles, formed by complexation of nucleic acids with biodegradable, cationic and magnetic beads by electrostatic interactions, result in efficient DNA delivery vectors in the presence of a locally applied magnetic field. The transport of these particles by an adjacent magnetic field leads to a high transfection rate with low toxicity and avoids harming the cell membrane (in contrast to biolistic transfection methods described in chapter 1.1.1.4). Transfection with DNA or siRNA occurs within 15 minutes to 24h by endosomal uptake on a broad range of cell types [13-17].

1.1.1.2. Electroporation

Local application of repeated short electric pulses by small electrodes increases the permeability of the cell membrane and therefore dramatically increases the uptake rate of DNA directly into the cytosol [18, 19]. Transfection by electroporation is effectively applied for *in vitro* transfection approaches for eukaryotic cells, especially in the case of hard to transfect cells [20], whereas the transfection efficacy varies depending on the targeted cell type. Advantageously, the electric pulses can be applied locally to the desired target cells or tissue, avoiding unintentional systemic side-effects. *In vivo* application has reached several promising results concerning the transfection efficacy [21, 22], even in the transfection of

neuronal cells in the mammalian brain [23]. But there are still some attributes required to be optimized, like cell and tissue damages due to the applied electric pulses.

1.1.1.3. Sonoporation

Beside the electroporation technique, the mediation of DNA delivery by ultrasound offers a promising and safe technique with a broad range of possibilities for clinical applications [24]. Depending on the settings of the sonoporation equipment (Table 1), an increased permeability of the eukaryotic cell membrane is achieved [25-29]. Additionally, for an enhanced delivery of macromolecules like therapeutic DNA vectors, the introduction of microbubbles, which are cavitated by the ultrasonic pulses, show significantly enhanced results concerning gene delivery [30]. In contrast to the delivery of macromolecules by electroporation (1.1.1.2), sonoporation offers a non-invasive and a more gentle technology for an efficient delivery of macromolecules *in vivo*.

Insonating acoustic pressure	0.05 to 3.5 MPa
Pulse duration	4 to 32 µs
Pulse frequency	0.5 to 5.0 MHz
Pulse repetition frequency	10 to 3000 Hz
Insonation time	0.1 to 900 s

Table 1: Parameters of sonoporation. Changes in the settings can significantly influence the efficiency of gene delivery [26]

	Therapeutic/reporter gene / additional components	*In vitro / in vivo*	Effect	Ref
Skeletal muscle of mice (transcutaneous)	human bone morphogenetic protein-2	*In vivo*	Osteoinduction	[31]
CHO, HEK293, NIH3T3	VEGF gene, branched PEI (25 kDa)	*In vitro*		[24]
Mice	VEGF	*In vivo*	markedly increased skin blood perfusion and CD31 expression -> Accelerated wound closure	[32]
Chicken embryos	Reporter constructs: GFP, lacZ	*In vivo*	efficient exogenous gene transduction and expression with lower damages to embryos	[30]

Table 2: Examples of successful approaches using ultrasonic gene delivery (sonoporation)

in vitro and *in vivo*

1.1.1.4. Ballistic gene delivery (gene gun)

Ballistic gene delivery methods offer a quick, contact-free and easy to use application by bombardment of cells with gold particles conjugated with exogenous DNA, which functions as biolistic DNA bullets [33, 34]. The gold particles conjugated with macromolecules are accelerated via helium gas pressure. Initially designed for efficient transfection of cells of plant tissues [35-40], this mechanical transfection method gain more and more popularity for the application of *in vitro* transfection of DNA and other macromolecules in a broad range of cells (Table 3) [41, 42]. However, the transfection efficiency is cell type dependent and shows the highest efficacy if applied on skin cells.

To date, different specific modifications and optimizations of the ballistic gene delivery method have been proceeded to adapt the gene delivery method to a broad range of applications [33, 34, 41, 43]. Moreover, gene transfer by ballistic methods gains more and more popularity for anti-tumor treatment. For affirmation, local ballistic administration of cytokine genes into tumor-bearing animals to suppress tumor growth and indicate tumor degradation is demonstrated to be a promising alternative application spectrum [44-46]. Furthermore, direct ballistic gene transfer shows great success in the immunization and vaccination of mice. In fact, an increase of the immune reaction according to the introduction of viral and bacterial genes (V antigen of Yersinia pestis or the E2 glycoprotein of Venezuelan Equine Encephalitis (VEE) virus [47] and immediate early protein of pseudorabies virus [48]), as well as vaccination of turkeys by introducing plasmids encoding the gene for the major outer membrane protein of *Chlamydia psittaci* [49], demonstrates the high potency of ballistic gene delivery approaches. Moreover, ballistic introduction of exogenous genes encoded for viral structural proteins [50] or mimotope genes (molecular mimicry) [51, 52] shows promising effects in the immunization of mice.

The following table lists examples successful delivery of macromolecules into mammalian cells using ballistic methods.

Model	Appl.	Reporter/therapeutic gene	Efficiency	Ref
Yeast mitochondria	*In vivo*	mitochondrial oxi3 gene	Complementation of oxi3 gene	[53]
Mouse, rat	*In vivo*		positively transfected skin, muscle and liver tissues of mouse and rat	[54]
Mouse skin and liver	*In vivo*	human Beta-actin promoter + luciferase gene	10-20% of cells transfected, luciferase was detectable over 14 days	[55]
Cultured inner ear sensory epithelia cells	*In vitro*	GFP tagged cDNA of beta-actin, whirlin and myosin XVa		[56]
Neurons (rat hippocampal cells)	*In vitro*			[57]
Yeast mitochondria	*In vivo*			[53]
Drosophila embryos	*In vivo*		Transient expression of DNA	[58]
Rat brain tissues primary cultures of fetal brain tissue Neuron and glial cells Freshly excised and bombarded fetal brain tissues (*ex vivo*)	*In vitro / ex vivo*	luciferase (luc) gene	luciferase detectable for up to two months	[59]
Mouse and rat skin, muscle and liver tissues of	*In vitro / in vivo*	chloramphenicol acetyltransferase, Beta-galactosidase		[54]
Dictyostelium discoideum (slime mold) vegetative AX2 cells	*In vitro*		2500 clones/µg DNA	[60]
Caenorhabditis elegans	*In vivo*			[61]
Mouse	*In vivo*	Transfer of TNF-alpha, IFN-g , IL-2, IL-6: reduced subcutaneous tumor growth in mice		[44]
Mouse	*In vivo*	Tested cytokines: IL-2, IL-4, IL-6, IL-12, TNF-alpha, IFN-g, GM-CSF. superior antitumor activity of interleukin-12 in mice bearing an intradermal murine tumor		[45]
Rabbit synovial fibroblasts (HIG-82)	*In vitro*			[62]
Corneal epithelium. Cornea of anesthetized rats.	*In vivo*		> 90% of the cells transfected	[63]
Cultured rabbit endothelial cells	*In vitro*			[64]
Rat liver	*In vivo*	DNA		[65]
Lamprey, brain neurons	*In vivo*	DNA (Beta-Gal)		[66]

Epidermal cells (skin transfection), mouse	*In vivo*	IL-12 cDNA Or Beta-Gal		[46]
Leech central neurons	*In vivo*	RNAi / pDNA		[67]
Hippocampal neurons				[68]
Human embryonic kidney 293 cells (HEK293) Whole brain	*In vitro* *In vivo*	Dye (cell labeling)	visualized in minutes	[69]
Fish cerebellum				[70]
Mammalian neurons				[71]
Immunization/vaccination				
Immunization: Turkeys	*In vivo*	major outer membrane protein (MOMP) of an avian Chlamydia psittaci strain	Protection of turkeys against Chlamydia psittaci	[49]
Vaccination/immunization Mice	*In vivo*	intra-dermal or intra-muscular, introduction of gene: V antigen of Yersinia pestis or the E2 glycoprotein of Venezuelan Equine Encephalitis (VEE) virus	Boost of IgG levels (increase of immune reaction)	[47]
Immunization: Mice	*In vivo*	Pseudorabies virus (PrV) immediate early protein (IE180)	immune response against PrV	[48]
Immunization: Mice	*In vivo*	cDNA encoding structural proteins (nucleocapsid) of the Rift Valley Fever virus	Immunization	[50]
Immunization Mice	*In vivo*	by introduction of mimotope genes (molecular mimicry)	Induction of IgG antibody response	[51]
Immunization: Mice Antigen-specific immunotherapy	*In vivo*	glutamic acid decarboxylase 65 (GAD65)	Treatment of Type 1 diabetes (T1D)	[52]

Table 3: Examples of an efficient delivery of molecules into mammalian cells by ballistic methods

1.1.2. Polycations

In general, transfection studies in which DNA was applied alone showed weak transfection efficiency independently of the targeted cell and tissue type *in vitro* and *in vivo,* respectively. Different reasons play an important role for this condition: Firstly, nude DNA molecules are easily degraded by nucleases out- and inside the cells, secondly, the long non compact shape of DNA molecules are sterically not adequate to enter target cells by endocytosis. Thirdly, the overall negative charge of the DNA backbone causes repellence from the negatively charged membrane surface of eukaryotic cells. And fourthly, a physical concentration of DNA on the cell surface (molecular crowding) in order to facilitate the uptake by endocytosis is

not given. To avoid the described situations, additional components have to be included in order to protect the DNA against degradation, decrease or entire neutralize the negative charge and convert the DNA structure into a sterically more applicable form.

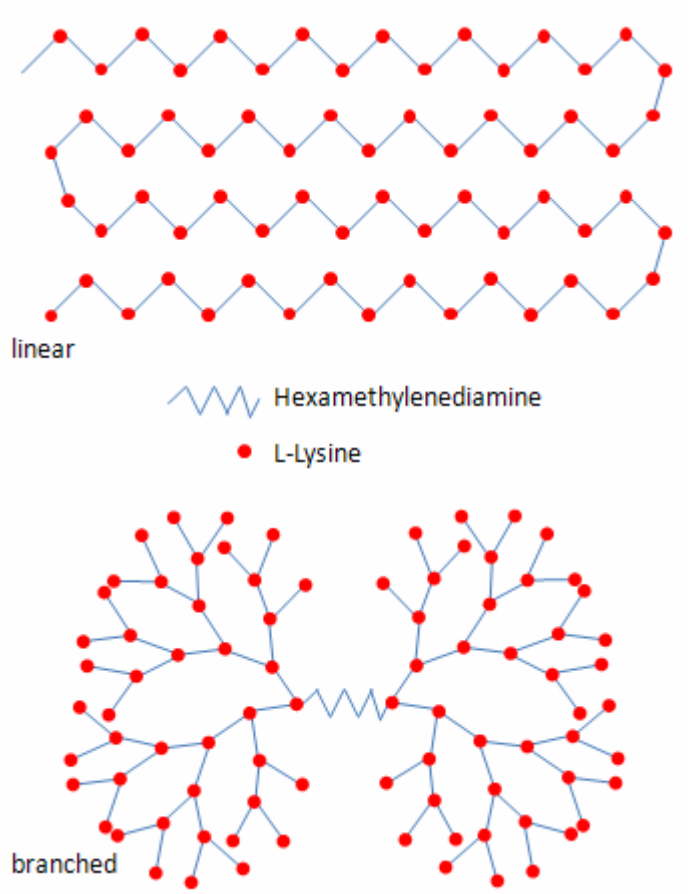


Figure 2: Schematic illustration of linear and branched poly-L-lysine

By complexing free DNA with positively charged carriers (polycations or cationic lipids), all of these conditions can be attained at once in order to guarantee a more efficient delivery of exogenous DNA into the target cells. Concerning the net surface charge, complexation of DNA leads to an overall neutral or positive surface charge and allows the DNA:carrier complex to interact with the target cell membrane by electrostatic interactions in order to enhance the cellular uptake through the endocytotic pathway [72]. Furthermore, the compact structure of the DNA particles is sterically advantaged for the uptake by endocytosis. And moreover, the complexation protects the bound DNA against the digestion by nucleases. The classical and most promising DNA carriers include the positively charged polyethyleneimine (PEI) [73], Poly(lactic-co-glycolic acid) (PLGA) [74] and poly-L-Lysine (PLL) with consists of homogenously repeats of the highly positively charged amino acid L-lysine. The size of these cationic macromolecules varies from 5 to 50 kDa. Moreover, the structure can be divided into branched (dendritic) and linear forms, respectively (Figure 2). It has been observed that branched polycationic structures can condense DNA more efficiently than linear forms [75, 76], but were less potent in transfecting eukaryotic cells. However, branched polycationic

macromolecules score to be less toxic than their linear forms, which in turn is an important factor concerning *in vivo* applications [77, 78].

Nevertheless, different behavior and characteristics in complexation, cytotoxicity, release and uptake of the complexed DNA can be achieved by altering the size [79], the structure and the molecular ratio [80] of DNA and polycationic carrier, and finally by additional chemical and structural modifications, respectively (Table 4) [75].

1.1.2.1. DNA condensation and N/P ratio

Concerning cell transfection efficacy, the formation of DNA condensates determines the future properties and behavior of the complexed particles. Alterations in the efficiency of DNA condensation by changing the N/P ratio influence cytotoxicity, uptake efficiency, DNA protection potential and release kinetics of DNA:polycation particles and is therefore considered to be an important factor in influencing the transfection efficiency.

Due to positively charged amino groups of polycationic macromolecules, ionical interactions with negatively charged phosphate groups of the DNA backbone leads to DNA condensation into small DNA:polycation particles [73, 76, 81, 82]. For estimation of the ionic balance of polycation and DNA, the N/P ratio gives a good benchmark for the relative amounts of the positively charged nitrogen groups of the polycation (N) and the negatively charged phosphate groups of the DNA backbone (P). By indicating the N/P ratio, an approximate value of the relativeness of DNA and cationic agent, and therefore the condensation behavior, is given [73, 83].

Complete complexation of polycations with DNA is observed to occur at N/P ratios between 1 and 4, forming particles with a neutral net charge [73, 80, 84]. In addition, the molecular weight of polycations is direct proportional to the DNA condensation efficiency. Decrease of the molecular weight leads to a less effective DNA binding activity [75]. However, small molecular weight PLL (10–30 residues) have still sufficient potential for DNA binding, and

advantageously form larger DNA particles with decreased toxicity compared with DNA particles complexed with high molecular weight PLL [72, 85].

1.1.2.2. Properties of polycationic carriers complexed with different types of DNA

Comparative studies with application of different DNA types (circular and linearized plasmid DNA, small oligonucelotides) imply that the uptake of the complexed particles is independent of the type of the DNA, but circular DNA was more active to get expressed inside the cells [80]. Nevertheless, condensation of small single-stranded DNA molecules with PLL leads to smaller complexes and increased gene delivery in comparison to double-stranded DNA [86].

1.1.2.3. Cytotoxicity (+ modifications to decrease toxicity)

Cytotoxic and immunogenic effects of polycationic DNA carriers are of imminent importance in particular for *in vivo* applications [73, 87-89]. Toxicity analysis reveal that non-modified cationic gene delivery carriers induce production of cytokines [90], and moreover, comparative studies in mouse fibroblasts rank PEI as the most toxic component followed by PLL and poly(diallyl-dimethyl-ammonium chloride) [91]. For affirmation, additional toxicity studies with PEI conjugated with DNA and PEI alone demonstrate an alteration of the gene expression pattern *in vitro* [92] and an activation of Th1/Th2- and adaptive immune responses *in vivo* [93]. Interestingly, PEI complexed with DNA shows a higher immunogenicity compared to PEI alone.

In conclusion, as rule of thumb, the transfection efficiency is inverse proportional to the toxicity [88, 94]. Wadhwa, M.S., et al. demonstrate lower cytotoxicity by decreasing the molecular weight of PLL. Moreover, 13–18 lysine residues have shown to have sufficient potential to bind DNA and form microparticles with decreased cytotoxicity [85]. Based on these results, additional modifications on polycations have demonstrated to lower their toxicity (Table 4) [95-97].

1.1.2.4. Non specific interactions with cells and proteins (blood components)

The transfection efficiency with cationic polymers (PEI and PLL) and lipids (DOTAP) is limited through the high ionical interaction potency of DNA carriers with serum and tissue components [98-100], especially with bovine serum albumin (BSA), lipoproteins, macroglobulin [89] and erythrocytes [101-103].

Unlike for *in vitro* applications, the avoidance of serum components in order to prevent non specific crossreactions, is not feasible for approaches *in vivo*. Therefore, interactions of the DNA carrier with circulating blood components have to be reduced by shielding the net positive charge of the polycationic carrier in order to achieve an efficient cell transfection. But by shielding the net charge, also the electrostatic attraction and adhesion with the negatively charged cell membrane is attenuated at the same time. For this reason, additional surface receptors or ligands have to be introduced into the vector system to ensure specific attachment onto the cell surface for an efficient cellular uptake [84]. As example, a promising DNA carrier arises by covalent attachment of polyethyleneglycol (PEG) onto the surface of cationic particles. The introduction of PEG (PEGylation) results in shielding the particles from non specific interactions with blood components and extended half life through stabilization of the microparticles, which transforms the cationic particles in a more applicable vector for systemic and local gene delivery *in vivo* [75, 84, 97, 101]. Additionally, a lower toxic effect is achieved (Table 4) [95, 96].

1.1.2.5. Protection of DNA against degradation by nucleases

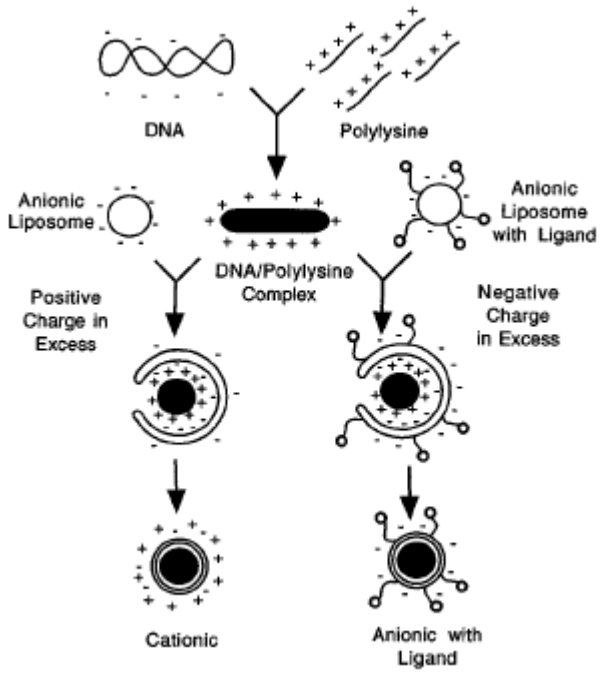


Figure 3: Formation of cationic and anionic microparticles by combining polycations with cationic lipids [104].

One additional limiting factor hindering an efficient gene delivery into target cells represents the degradation of exogenous DNA by intra- and extracellular nucleases. Based on these facts, the protection of exogenous DNA during gene delivery guarantees a safer transport of exogenous DNA into the nucleus of target cells. The increase of the probability of DNA to reach the cell nucleus provides essential criteria in the improvement of the transfection efficiency. Regarding this fact, polycations show promising results in DNA protection after complexation following treatment with nucleases [84, 105-108]. Furthermore, additional DNA protection can be achieved by surface modifications of the particular DNA carrier systems, like the conjugation of asialoglycoproteins to PLL macromolecules (Table 4) [109]. Observations with small PLL (7-30 and 18 lysine residues, respectively) have demonstrated their high DNA protection potential against digestion by nucleases for more than 45 minutes, whereas free DNA was already degraded after the first minutes following nuclease treatment [110]. An additional observation demonstrates an increased protection of DNA in PLL:DNA particles by addition of lipid membranes forming cationic or anionic lipoparticles (Figure 3) [111].

1.1.2.6. Transfection of cells using polycations (and modifications to enhance transfection)

An efficient uptake and expression of exogenous genes *in vitro* and *in vivo* is dependent on an efficient DNA protection against degradation by nucleases in the blood circulation and cell-cell compartments [110], the rate of condensation and release kinetics of DNA bound to the polycation, and their rate of interactions with blood components, cell membrane and extracellular matrix, respectively [84].

Polycationic particles complexed with DNA are able to transfect primary cells [112-115] and cell lines [107, 116, 117] *in vitro* and *in vivo* [98, 115, 118], respectively, whereas the transfection efficiency varies depending on the cell and tissue type [73, 119]. As a benchmark, the internalization of polycationic particles occurs within 10-30 min, whereas maximum uptake is reached after 2 hours [116, 120]. Based on this, different chemical structures of the DNA carrier effect different physicochemical behavior and can either in- or decrease the cellular uptake and therefore influence the transfection efficiency [121]. By increasing the positive net charge and the particle size, the interaction with the cell membrane and the proteoglycans on the cell surface and therefore the uptake rate of particles is enhanced [86, 110, 119]. In conclusion, the transfection efficiency is influenced by the particle size and the net surface charge which can be altered by changing the DNA/polycation ratio or the molecular weight of the polycation (the higher molecular weight, the smaller the particle size) [94, 122]. But the ratio of DNA and polycation, and therefore the rate of DNA condensation, has to be determined carefully. High excess of positively charged macromolecules can improve the uptake and transfection efficacy by enhanced electrostatic binding with the negatively charged membrane surface of the target cell[86], but hinder efficient access to the introduced DNA. Additionally, high amounts of polycations can protect its complexed cargo DNA against degradation by nucleases more efficiently [75, 110].

Condensation analysis of DNA with linear PLL have shown a more efficient DNA complexation and uptake in comparison with dendritic polypeptides [75, 123], but on the other hand a less efficient gene expression assumably due to too tight interactions and

therefore low DNA release rates of the PLL carrier [90]. In contrast, no decrease in the transcription rate of bound DNA could be observed when DNA was closely attached to PEI [124].

1.1.2.7. Properties of polycations for lysosomal escape (proton sponge)

Uptake of PEI/DNA particles into endosomes in target cells occurs within 10-20 minutes [116, 120]. But in contrast to the particle uptake, lysosomal release is demonstrated to be the rate limiting step in the cell transfection with PEI/DNA particles [124]. Entrapment and degradation in the lysosomes of target cells highly limits the efficiency of gene delivery and leads to a considerable decrease in the number of cells expressing the introduced gene [116]. This data demonstrate that transfection efficiency is not only dependent on rate of DNA uptake but also on the probability to escape from the endosomes [125].

Concerning cell transfection with polycations, several groups postulate the so-called “proton sponge hypothesis” observed in cell transfection approaches using PEI as DNA carrier. Unlike PLL, PEI shows the capacity to buffer the acidic lysosomal pH, causing water influx by osmotic forces leading to a burst of the vesicles [75, 94, 106, 124, 126-128]. Probably, the amino groups, which represent the main molecule group of PEI, are responsible for the pH buffering ability [115].

1.1.2.8. Modifications to enhance transfection

Transfection efficiency with DNA condensed by polycations is still poor due to the low endosomal escape and access of complexed DNA, and additionally due to high cross reactions with blood components [75]. To increase the efficacy, several modifications of polycationic carriers have led to improved abilities of the DNA carrier (Table 4).

Modification	**Description**	**Effect**
PEGylation	introduction of the hydrophilic polymer PEG, commonly used in *in vivo* applications	Sterical stabilization of the complex [75, 129, 130]
		Decrease the toxic effect of the polymer complex (comparatively to PLL and PEI homopolymer) [95, 96,

		130]
		Better protection of DNA against degradation by nucleases (relatively to PLL homopolymer Mw: 25,700) [96]
		can condensate DNA into long filamentous structures (∅ 6-20 nm), more efficient [121]
		Protection of DNA from DNase I digestion for more than 60 minutes [131]
		lower cytotoxicity and higher transfection efficiency in COS-1 cells 3-fold higher transfection efficiency in muscle *in vivo* [97]
		reduced interaction with blood components, extended circulation in blood stream [101, 103, 132] Increased solubility [133, 134]
Asialoglycoproteins		Enhanced resistance of DNA to degradation by nucleases [109]
Serine residues	Introduction of 25 mol% serine residue to PLL	(slightly enhanced gene expression) [135]
Pluronic-grafted PLL		(2-fold increase in transfection efficiency, no difference in cytotoxicity) [136]
Iron	poly-L-lysine modified iron oxide nanoparticles (IONP-PLL)	[137]
Galactosylation		binding on asialoglycoprotein receptor → receptor-mediated endocytosis [86]
Arginines, histidines	Exchange of terminal lysines of dendritic PLL with arginines and histidines	[138]
Conversion of PLL into amphiphilic vesicle forming polymers		reduction of toxicity [87]
Palmitic acid-grafted PLL	PLL substituted with palmitic acid	slightly decrease DNA binding efficiency but enhance cell binding to bone marrow stromal cells (BMSCs) resulting into a significant higher polymer uptake and around fivefold higher transfection efficiency relatively to transfection with PLL alone [139]
Oligodeoxynucleotides (ODN) (14-20mer)	Covalent linkage of ODN to PLL	Inhibition of cellular and viral gene expression at the molecular level [140]
Folate-pEG-Polymer 1-pLL/DNA complex		Cell specifity (cell targeting) [141]
Dendritic poly(l-lysine) of the 6th generation (KG6)		Increased half-life in blood stream, low toxicity [142]
Transferrin		Tumor cell targeting in systemic application [102, 143]
		Shielding positive net charge of polycationic particles, cell targeting through attachment of transferrin on

		transferrin receptor [101, 144] reduced interaction with blood components, extended circulation in blood [101]
		Inhibition of cross reactions of polycations with erythrocytes [101, 103] Reduced cytotoxicity of polycations *in vivo* [102]
RGD domain		Adhesion onto the cell membrane [145]
Pluronic 123 (polyether)		reduced lung gene expression, increased liver expression *in vivo* [146]
Poly-N-(2 -hydroxy-propyl)methacrylamide (pHPMA)	PLL	Net negative surface charge, increased solubility, lower toxicity and a negative surface charge. Reduced the interaction with blood components [144]
Poloxamer		increased gene expression in muscle, probably more efficient diffusion throughout the tissue [147]
Folate-pEG-Polymer 1-pLL/DNA complex		Cell specificity (cell targeting) for systemic administration *in vivo* [141]

Table 4: Commonly used structural modifications of polycations in order to increase the transfection efficacy

1.1.3. Liposomes/cationic lipids

Liposomal gene delivery vehicles consist of cationic lipid particles (normally mixed with neutral lipid components) forming an efficient DNA delivery tool. The combination of different lipid components (Table 5) leads to different chemical behavior and can therefore improve the properties of the particular delivery vectors, respectively [148]. By entering the target cell via the endocytotic pathway [149], gene delivery vectors based on lipid formulations offer a potent method of introducing macromolecules like DNA into a broad range of eukaryotic cells. However, one limitation for an efficient expression of the introduced exogenous DNA using cationic lipids is the aggregation of the cationic lipids into large perinuclear complexes in the cytosol hindering the encapsulated DNA to reach the cell nucleus [149].

DMRIE	N-[1-(2,3-dimyristyloxy) propyl]-N,N-dimethyl-N-(2-hydroxyethyl)ammonium bromide
DOPE	dioleoyl phosphatidylethanolamine
DOTMA	N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-trimethylammonium chloride
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
DODAC	dioleoyldimethylammonium chloride
DOSPA	*2,3-dioleyloxy*-N-[*2*(sperminecarboxamido)ethyl]-N,N-dimethyl-1-

	propanaminium trifluoroacetate
DODMA	(*2,3-dioleyloxy*)propyl)-N,N-dimethylammonium chloride

Table 5: Commonly applied liposome formulations

1.1.4. Silica nanoparticles

One potential alternative of gene transfection using polycations and cationic lipids are silica (SiO2) nanoparticles. Due to their high DNA and cell surface binding capacity by electrostatic interactions, cationic silica nanoparticles exhibit a high transfection potential of pre-complexed DNA by local concentration onto the target cell surface *in vitro* [150-152] and *in vivo* [151, 153], even in the presence of high serum concentrations. Due to the low potential to interact with and therefore get inhibited by serum proteins, gene delivery by silica nanoparticles has a high potential to be successful in *in vivo* applications. By additional combination with classical polycation based transfection reagents, a significant enhancement of the transfection efficiency was achieved through high endosomal uptake of the complexed silica particles [154, 155]. Additionally, SiO2 particles effectively protect complexed DNA from degradation by nucleases and feature low toxicity [152].

1.1.5. Receptor/ligand interaction

Although the application of delivery systems using ionical attraction for the adhesion to the target cell membrane have strong potential for the cell transfection *in vitro*, the *in vivo* administration of such particles leads to unwanted electrostatic interactions with the blood components. Therefore, specific interactions of DNA complexed particles with the target cell membrane, for example by receptor/ligand interactions, ensure avoiding unwanted ionical interactions with blood and tissue components and can specifically target only the cells and tissues of interest. The following chapters list possible and commonly used receptors for cell adhesion.

1.1.5.1. Transferrin

Transferrin, an 80kDa serum glycoprotein, is mainly responsible for the iron transport into the cells. Due to its two iron binding sites, it can bind iron ions from serum with high affinity. After binding, the transferrin-iron complex is able to bind to the transferrin receptor expressed on the surface of cells (Transferrin receptor 1 (TfR1) is expressed in all cell types, Transferrin receptor 2 (TfR2) is only expressed in the liver). After binding, the receptor ligand complex is transported into the cell by the receptor mediated endocytic transferrin cycle. Due to conformational changes caused by the acidic pH in the endosomes, the two bounded iron ions are released and the receptor is transported back to the cell surface and ready for the next iron cycle [156]. Because of to the high expression of the transferrin receptor on the surface of heavy proliferating cells such as tumor cells, transferrin-coated liposomes are successfully used for cell targeting applications in cancer therapies [157].

For systemic gene delivery of positively charged DNA carriers *in vivo*, transferrin molecules effectively shield the positive surface charge and therefore decrease cross reactions with negatively charged serum components. Therefore, surface linked transferrin (Tf)-lipoplexes can obtain a good transfection efficiency even in the presence of high serum concentrations (up to 60% of FCS) [158]. The transferrin coated particles find additional application in the field of gene silencing, where siRNAs complexed with transferrin have shown inhibition of luciferase expression in cortical neurons [159].

1.1.5.2. Invasin

Invasin, a cell surface protein of the enteropathogenic bacteria *Yersinia*, promotes efficient cell invasion by adhesion onto cell surface proteins expressed on the majority of mammalian

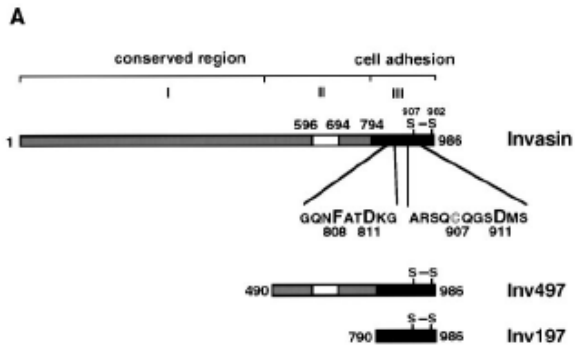


Figure 4: Invasin and truncated forms sufficient for the receptor-mediated uptake by eukaryotic cells (adapted from [160])

Cells [161, 162]. By mimicry of fibronectin, invasin is able to bind to several members of the superfamily of the mammalian cell surface receptor integrin ($\alpha_5\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$) expressed on the surface of most eukaryotic cell types in order to get access into the host cell by forced receptor mediated endocytosis [163]. It has been demonstrated, that a carboxy-terminal fragment of invasin (192 amino acids) is sufficient for cell adhesion and low rate uptake [164], whereas the last 497 carboxy-terminal amino acids are necessary to promote high efficient bacterial entry (Figure 4) [165]. Based on this, Dersch et al demonstrated the uptake of invasin coated silica particles in Hep-G2 cells [160].

1.2. Overcoming the lysosomes

Because exogenous DNA is rapidly degraded by lysosomal nucleases, the escape of DNA vectors from the lysosomal compartments is an important step for efficient gene delivery [128, 166, 167]. Several approaches to lyse or neutralize lysosomal vesicles, or promote a fusion with its membrane, are introduced below.

1.2.1. Lysis of lysosomes

The lysis of the lysosomes promises the most efficient way to ensure a safe delivery of macromolecules into the cytosol of eukaryotic cells. Lysosome disrupting potential of cationic lipids and PEI (1.1.2.7) has been postulated by several groups [75, 94, 106, 124-128]. But, due to their high toxicity and unwanted cross reactions with tissue and blood

components, additional investigations are needed to improve these polycations into a more applicable form.

1.2.1.1. Photoinduction

Photochemical transfection is based on the light activation of photoreactive compounds (aluminum phthalocyanine (AlPcS2a)) previously integrated into the lysosomal membrane. Upon activation by illumination of cells preincubated with the photoreactive compound, the membrane structure is disrupted, releasing the endocytosed DNA into the cytosol [168-171]. Several groups have demonstrated a significant enhancement of transfection mediated by cationic polymers by photochemical transfection [168, 172]. One additional advantage of photochemical transfection features the possibility of a site specific activation of lysosomal disruption, which in turn can be of interest in local gene therapy approaches *in vivo*.

1.2.1.2. Amphipathic (pore forming) peptides: GALA, KALA, Melittin

Due to their endosome disrupting activity, amphipathic peptides (peptides with both, a hydrophobic domain and polar head group) are adopted for gene delivery in order to overcome the lysosomal barrier [173, 174]. Several tested amphipathic peptides derived from different origins or designed synthetically, give promising results concerning their efficacy in delivering exogenous DNA fragments. The conformational structure of synthetic peptides like GALA (glutamic acid-alanine-leucine-alanine) and KALA (lysine-alanine-leucine-alanine) allows sterical adjacency of glutamic acid and lysine residues, respectively. Under acidic conditions (pH = 5), the protonation of the glutamic acid and lysine residues leads to a mutual repulsion of the cationic chains and hence to a conformational change to an amphipathic alpha-helix. The following exposition of hydrophobic helices promotes the interaction and following rupture of the uncharged lipid bilayer membrane [175-178].

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ), an example of a naturally derived amphipathic peptide, is of interest for gene transfer approaches due to its pore-forming activity. The amphipathic peptide (non polar N terminus and polar C terminus [179]) and main

component of the bee venom [180], shows membrane disrupting activity by assembling to a tetrameric structure inducing hydrophobic insertion into the lipid membrane within milliseconds in order to form trans-membrane ion channels (impermeable for water [181]). The subsequent efflux of K^+ ions leads to cell death [182-188].

In gene delivery approaches, melittin (and derivates [189]) attached to a polycationic DNA carrier (PEI) strongly increases the transfection efficiency of complexed DNA compared with transfection efficiencies of DNA attached to PEI alone. Additionally, due to the possible nuclear localization sequence cluster (KRKR) at the C-terminus, an active transport into the nucleus mediated by melittin was observed [190, 191]. Moreover, conjugation of melittin with the DNA carrier poly-L-lysine shows an up to 1800-fold higher expression level of the introduced exogenous in comparison to the DNA transfection with unmodified poly-L-lysine [192]. However, the high cytotoxicity arising from the membrane-disrupting activity of melittin limits their capable range of applications in gene delivery approaches. By inducing growth arrest and apoptosis on human hepatocellular carcinoma (HCC) cells, introduction of melittin shows a promising approach for the treatment of HCC [193], and probably also for other types of tumor cells.

But to increase the possible range of applications of melittin, present modifications to decrease its cytotoxicity and additionally introduce pH-dependent activity have shown promising results for a successful application for gene transfer approaches [192, 194].

1.2.1.3. Listeriolysin O

Due to its facultative anaerobic lifestyle, the Gram positive bacteria *Listeria monocytogenes* tends to attain the host cell cytosol for intracellular growth and proliferation. In order to escape lysosomal entrapment and gain access to the cytosol of eukaryotic cells, lysosomal degradation after engulfment has to be avoided. Therefore, secretion of the hemolytic protein Listeriolysin O (LLO) causes membrane perforation and releases the bacteria from the lysosomes [195-197]. The expression and secretion of the LLO, encoded by the bacterial *hlyA* gene [198], is responsible for the invasiveness of *L. monocytogenes*. Based on this, the pathogenic ability of LLO was demonstrated by transposon mutagenesis [197, 199] and

complementation analysis of the hlyA gene, where the invasiveness and pathogenicity of the bacteria was recovered in mice [200].

The main difference between LLO and other hemolytic proteins such as streptolysin or perfringolysin is the advantage of the pH dependent activity of LLO. In order to prevent the host cell wall from damage, the hemolytic activity of LLO is inhibited in cytosolic non acidic conditions. For affirmation, activity analysis with purified LLO showed increased hemolytic activity at acidic pH which can be compared with lysosomal conditions [201-204]. Additionally, *in vitro* studies with pH-sensitive liposomes packed with LLO and a fluorescence dye [205] or toxin [206], respectively, and bacterial cells expressing LLO [207] confirm these observations.

To conclude, by application of LLO in gene transfer approaches, lysosomal degradation can be bypassed and therefore a more efficient delivery of therapeutic DNA vectors, proteins or toxins *in vivo* can be achieved.

1.2.2. Fusion with lysosomal membrane

By fusion of the artificial constructed microparticles with the lysosomal membrane, the entrapped content of the particles can be directly delivered into the cytosol of the target cell. The following chapters list commonly used auxiliary domains for an efficient fusion with the target membrane.

1.2.2.1. Hemagglutinin (influenza virus)

Hemagglutinin (HA), a membrane bound glycoprotein of influenza virus, plays an important role in introducing viral genome into the host cell during the infection process [208, 209]. By binding of HA to sialic acids on surface receptors of eukaryotic cells, viral particles are endocytosed by target cells. Through conformational changes in acidic endosomal conditions, hydrophobic regions of the HA peptide are exposed forming amphipathic peptides. Following insertion into the endosomal membrane causes a fusion with the

membrane of the virus leading to an escape of the viral content into the cytosol (Figure 5) [210, 211].

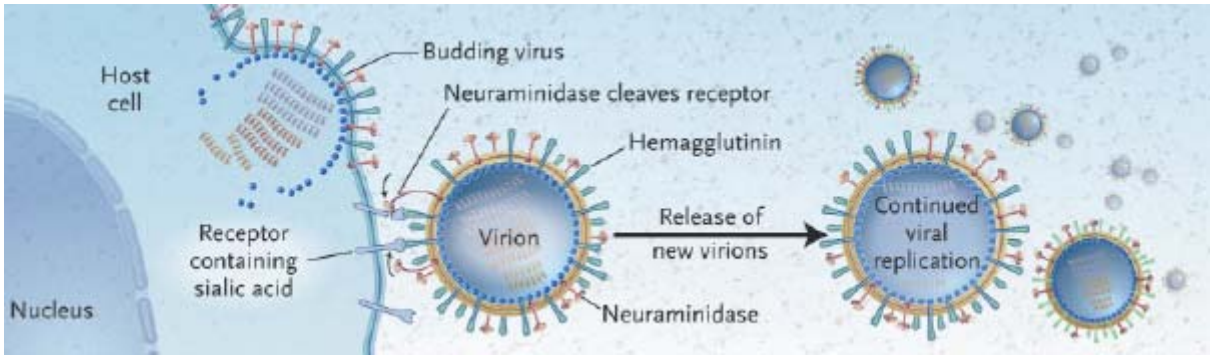


Figure 5: Life cycle of the influenza virus (adapted from [212])

In gene delivery applications, co-complexes of asialoglycoprotein and the N-terminal domain of hemagglutinin (HA-2) trigger the fusion of phosphatidylcholine vesicles and therefore strongly enhance the gene transfer into eukaryotic cells [210, 213-216].

1.2.3. Neutralization of lysosomes

By neutralizing the acidic conditions in the lysosomal compartments, two effects affecting an improved gene delivery can be attained. Firstly, the buffering of the pH prevents the fusion of late endosomes with lysosomes, and secondly inhibiting the acidic lysosomal nucleases preventing the degradation of the introduced DNA [167]. Table 6 lists substances influencing the delivery of macromolcules into eukaryotic cells.

Chloroquine	weak base that buffers acidic cellular vesicles	enhances the *in vitro* transfection of eukaryotic cells by non-viral vectors	[216]
Bafilomycin A1	Antibiotics	prevents the acidification of the vacuoles by inhibiting the vacuolar proton pump	[167, 217]

Table 6: Substances influencing the delivery of macromolcules into eukaryotic cells

1.3. Overcoming the nuclear membrane

Gene delivery into rapidly dividing cells is more efficient due to the dissolved nuclear membrane during the cell division [218-220]. But in the case of *in vivo* cell transfection, the majority of cells have very slow division rates. Therefore, many types of viruses have to import their DNA as well as other macromolecules into the host cell nucleus to ensure their replication and transcription. In most cases, the host cell´s own import machinery is utilized by targeting host import proteins by nuclear localization signals encoded on viral proteins in order to ensure active import of these macromolecules through the nuclear pore complexes [221, 222]. In contrast to viral vectors, non viral gene delivery methods show low transfection efficiencies *in vitro* and *in vivo*, not at least because of lacking efficient methods to overcome the nuclear membrane of non-dividing cells. Most introduced plasmid DNA remains in the cytosol [79, 172] and is degraded by cytosolic nucleases within 2 hours, whereas the time of the degradation is independent of the cell line or the type of plasmid DNA [108, 223]. Yamaizumi et. al. demonstrate that at least 1000-fold more DNA is required for an efficient expression of injected DNA into the cytosol in contrast to DNA injected directly into the cell nucleus [224]. Moreover, Labat-Moleur et al reported that only 1 of 100 plasmid DNA molecules injected into the cytoplasm of cells were able to reach the nucleus [225]. Furthermore, it has been demonstrated that the efficiency of the DNA import is higher in the case of applying linear DNA fragments smaller than 1,5kb [226]. Beside these observations, the transfection efficiency and level of gene expression is dependent on various factors such as plasmid size [227] and copy number [12]. Therefore, an increased copy number of plasmids inside the nucleus of eukaryotic cells leads to an increase in the gene expression [228].

To find solutions for this rate limiting step, an understanding in the molecular mechanism based on the nuclear transport is essential.

1.3.1. Nuclear pore complex (NPC) & nuclear transport pathways

In order to allow the passage of molecules through the nuclear membrane of eukaryotic cells, the double layer membrane of the nuclear envelope is perforated by basket-shaped nuclear pore complexes (NPCs) which consists of 8 assembled channels of around 10nm in diameter forming one single pore complex (Figure 6) [229-231], whereas the nuclear pore size is dependent on the cell type and the age of the cells [232]. These channel-like complexes allow the transmembrane transport of molecules either by passive diffusion or by active transport after interaction with the NPC. In order to allow free diffusion, macromolecules should not exceed a maximum molecular weight of 50 kDa, whereas the passage of molecules by diffusion is tended to be size dependent and not dependent on the molecular weight. Therefore, interactions with cytosolic components can decrease the diffusion rate dramatically [233, 234]. Macromolecules larger than 50 kDa (for example transcription factors) dock on the NPC with the help of specific adapter proteins in order to use the large central NPC channel for active signal-mediated transport through the nuclear membrane [234-236]. To activate the cell host transport mechanism, a translocation signal (nuclear localization signal) on the primary sequence of the protein is required in order to interact with shuttle proteins, which in turn can interact with the nuclear pore complex to convey the bounded protein through the nuclear membrane [229, 237-239].

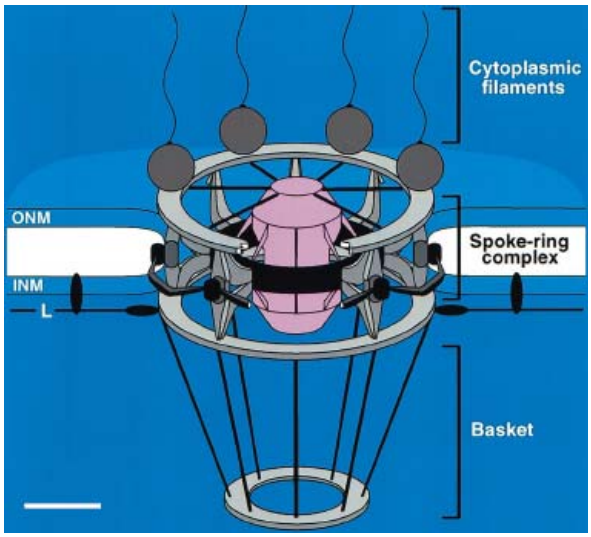


Figure 6: The nuclear pore complex: transport channel between nucleus and cytoplasm (adapted from [233])

Among several explored nuclear import mechanism, the importin pathway is presently the best described (Figure 7). After protein translation in the cytosol, proteins equipped with a nuclear localization signal that targets the importin pathway, bind tightly to importin α (karyopherin) following binding to the import mediator importin beta (adapter between importin a and NPC), whereby the protein complex can dock on the nuclear pore complex

and pass through the nuclear membrane by an active transport mechanism [240-245]. The observed nuclear accumulation occurs within 1 hour [246].

Inside, Ran-GTP, a nuclear G-protein, binds to the complex and leads to an affinity decrease of importin to its bound cargo protein. After the release of the protein, the RanGTP/Importin complex returns to the cytosol where Ran-GTP gets hydrolyzed by the GTPase activating protein (GAP) creating Ran-GDP. The hydrolyzed G-protein loses its affinity to importin and hence releases it into the cytosol where the cycle can start again [247-251] (more detailed information about import proteins and receptors are reviewed by H. Fried and U. Kutay [252]).

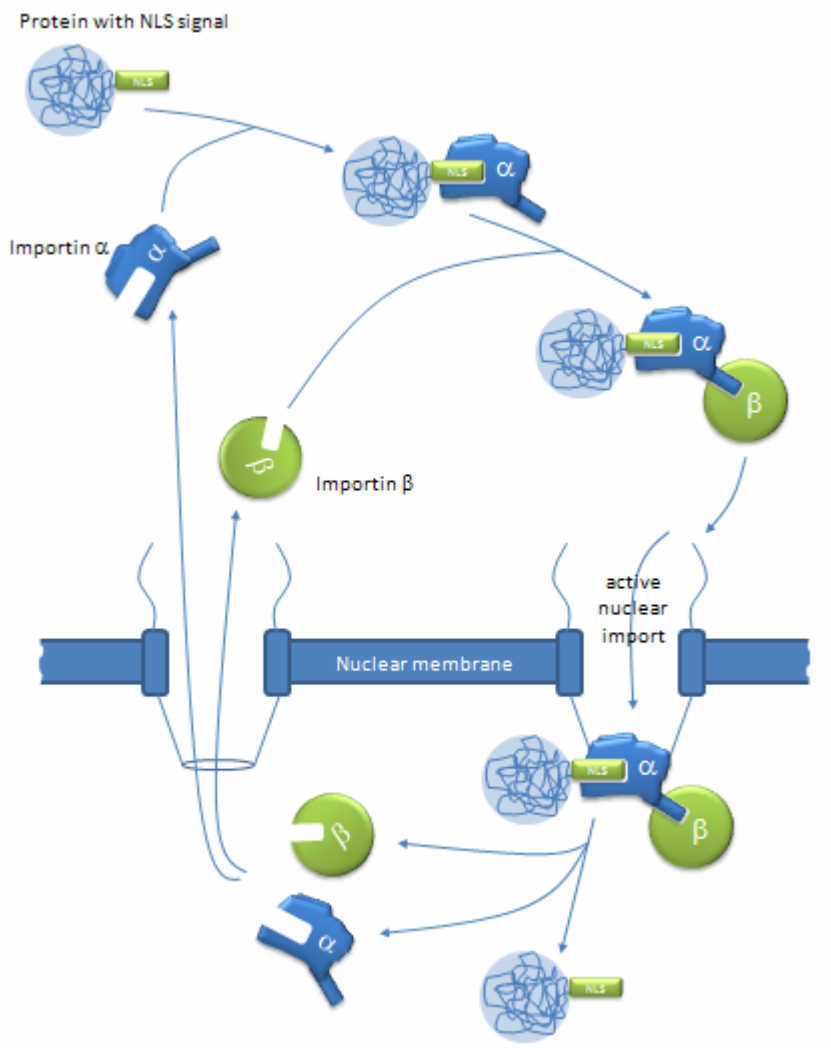


Figure 7: Importin pathway for active transport of NLS-tagged proteins into the cell nucleus

Among several tested nuclear localization sequences for their efficacy to direct proteins into the nucleus, the NLS derived from the large T antigen of simian virus 40 (SV40), PKKKRKV, seems to be the most potent nuclear import carrier even for huge molecules [253]. Additional efficiency tests showed an up to 3-fold increase of nuclear accumulation of NLS-tagged proteins by addition of purified peptides from bovine erythrocytes that bind NLS of the SV40 large T antigen [254]. Furthermore, gold particles (25nm) covered with NLS peptides or nuclear proteins could passage through the nuclear membrane with a high efficiency [255, 256].

By utilizing the importin pathway, NLS derived from SV40 is preferentially used for application in gene delivery approaches [257, 258].

1.3.2. NLS sequences

The nuclear localization signal is a short sequence tag consisting mostly of positively charged amino acids like lysine and arginine. Table 7 shows different nuclear localization sequences derived from different proteins and species (signal sequences listed below show the minimum of required essential amino acid sequence for the nuclear import, whereas additional amino acids flanking the NLS and the adjacent sterical conformation can affect the affinity of NLS to the nuclear import receptor [244, 259, 260]).

Protein	**Origin**	**Sequence**	**Recognition by**	**Ref.**
Large T antigen	SV40	PKKKRKV[261] CYDDEATADSQHSTPPKKKRKVEDPKDF ESELLS [262]	Importin α	[258, 261, 262]
Nucleoplasmin		Bipartite (AV)KR[PAATKKAGQA]KKKK(LD)		[263-266]
M9 domain of hnRNP A1 BIPARTITE	pre-mRNA/mRNA binding proteins in eukaryotic cells	(GNYNNQ)NQSSNFGPMKGGNFGGRS SGPYGGGGQYFAKPRNQGGY(GGS) YNDFGNYNNQSSNFGPMKGGNFGGRS SGPY [267]	Importin b-like shuttle protein transportin 1 (Transportin pathway[268, 269])	[267, 269-272]

Matα2 (transcription repressor)	Yeast	KIPIK	Importin β	[273]
Vpr	HIV-1	DTWTGVEALIRILQQLLFIHFRIGCRHSRI GIIQQRRTRNGA C-terminal	independent of the importin α/β or transportin pathway	[274]
Ad3 (fiber protein)	Adenovirus	AKRARLSTSFNPVYPYEDES hydrophobic domain FNPVYPY NPXY: receptor-mediated endocytosis NLS signal: KRARLSTSF		[275]
Histone H1			Importin β/importin 7 Nuclear protein histone H1 and HMG17 can condense DNA forming efficient cell transfecting complexes	[235, 276, 277]
Histone H2B	yeast	aa: 28-33 GKKRSKA	KapBeta1 [278, 279]	[280, 281]
RS domain	SR proteins (e.g. ASF/SF2 and SC35)	RS sequences (phosphorylated) arginine/serine (RS)-rich proteins	Transportin-SR [282] SR2 [283]	[282, 283]
NLS-H1 (Histone H1)			SV40 NLS linked to the carboxy-terminal domain of human histone H1(0) (DNA binding domain)	[277]
human immunodeficiency virus type 1 (HIV-1) Integrase		IIGQVRDQAEHLK	Interaction with importin alpha, competes with SV40 large T antigen	[284]
MAT alpha 2	S. cerevisiae	A 13 amino acid sequence part from alpha 2 is sufficient for nuclear localization		[273]
Vp2/3	SV40	GPNKKKRKL	Viral structural proteins	[246, 285]
pp65	Human Cytomegalovirus	CQPAAQPKRRRHRQDALPGPAIASTPK KHRG	Bipartite, Difference: both boxes can promote nuclear import, but stronger effect if together on sequence	[286]
HDAg Hepatitis Delta antigen	Hepatitis Delta virus	CKKDKDGEGAPPAKKLRMDQMEIDAG PRKRP		[287]
vp1B	SV40 (domain of VP1)	MKMAPTKRKGSAPGAAPKKPKC		[288]
BIB domain of rpL23a (aa: 32-74)	Yeast	VHSHKKKKIRTSPTFRRPKTLRLRRQPKY PR KSAPRRNKLDHY	transportin, Imp5, Imp7, Importin β	[289]
Histones H2A,	Yeast		Kap114p (yeast)	[290]

H2B			Importin 9 (human)	
hSRP1 alpha (NLS binding motif)	Yeast		a cytosolic receptor for both simple and bipartite NLS motifs	[291]
growth factor (LEDGF)/p75 protein	Human lens epithelium	GRKRKAEKQ	importin α, Interacting with human immunodeficiency virus type 1 (HIV-1) integrase	[292]
MAN1	Human	RRKP, RPRR		[293]
LAP2β	Human	PRKRVET		[293]
LAP1A	Human	PVGKRTR, RRQPRPQETEEMKTRRT		[293]
TMEM43/LUMA	Human	PAVKLRR		[293]
Emerin	Human	RRLYEKKIFEYETQRRR		[293]
LEM2	Human	PAQLRRR		[294]
SUN2	Human	RRRR		[293]
Heh1	Saccharomyces cerevisiae	PRRSRRA, RREKSASPMAKQFKKNR, RKKRK, KKRK, PRQKRHL		[295]
Asi3	Saccharomyces cerevisiae	KKPRVGKRKKR, RKKRDLNKYVTEKNYKK		[296]
Nem1	Saccharomyces cerevisiae	PKKPKAL, KKLIPKSVLNTQKKKKL		[297]
Heh2	Saccharomyces cerevisiae	KRKR, PKKKRKKR		[295]
Mps2	Saccharomyces cerevisiae	PRKK, KRKH		[298]
Ydl089w	Saccharomyces cerevisiae	PKKKK		[295]
Pga1	Saccharomyces cerevisiae	KKPR		
Spo7	Saccharomyces cerevisiae	PRRR, RRRK		[298]
Prm3	Saccharomyces cerevisiae	RKHKTTTSSTKSRTKSK		[299]

Table 7: Nuclear localization signals derived from different origins

1.3.3. Application of NLS for an increased transfection efficiency

Because most cells targeted in gene therapy approaches are non-dividing or slowly-dividing cells, the incorporation of nuclear localization signals (NLS), capable of mediating nuclear

import, can significantly increase the expression rate of the introduced exogenous DNA [226, 253, 258, 277, 300-303]. It has been demonstrated that only one nuclear localization sequence conjugated to DNA is sufficient to mediate the nuclear import [304]. As additional examples, increase of the transfection efficiency using conjugated NLS derived from SV40 was obtained with linear DNA molecules using either microinjection approaches [228], cationic lipids [271, 304, 305], polycations [306] or electroporation [307], respectively.

But in contrast, other observations showed no increase of NLS-conjugated transfection by using cationic lipids and polycations, respectively [303, 308, 309]. Direct introduction of exogenous DNA by microinjection showed no nuclear detection of the DNA, neither with linear DNA conjugated to NLS bearing peptides alone [309, 310] nor in combination with cationic polymers [303, 311].

1.4. Aim in this work

The aim of this study was to overcome the lysosomal degradation of introduced therapeutic DNA by disrupting the lysosomes of the eukaryotic target cells (principles described in section 1.2.1.3). For this purpose, the bacterial hemolytic protein LLO was expressed in *E. coli* und purified until homogeneity and applied directly to gene transfer systems to test the possible enhancement of the delivery of macromolecules in mouse skeletal myoblast precursor cells.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Wizard SV Minipreparation Kit	Promega	Madison, WI, USA
Wizard SV Gel and PCR Clean-up Kit	Promega	Madison, WI, USA
Hot Taq-DNA polymerase	PeqLab Biotechnologie GmbH	Erlangen, Germany
Endo-Free Maxiprep Kit	Qiagen	Hilden, Germany
DMEM high glucose	Sigma-Aldrich	Missouri, USA
FCS	Cambrex	East Rutherford, NJ
Trypsin	Sigma Aldrich	Missouri USA
PCR Primer	Invitrogen	Carlsbad, CA
Lipofectamine 2000	Invitrogen	Carlsbad, CA
Anti-poly-Histidine-PE Monoclonal Antibody	R&D Systems GmbH	Wiesbaden-Nordenstadt, Germany
ANTI-POLYHISTIDINE monoclonal antibody (clone 1) His-1 Peroxidase conjugate	Sigma-Aldrich	Missouri, USA
6X His tag® antibody (HRP)	Abcam	Cambridge, MA, USA
TA-cloning Kit (including pCR2.1 vector)	Invitrogen	Carlsbad, CA
pcDNA3 (vector)	Invitrogen	Carlsbad, CA
pEYFP (vector)	Clontech	Palo Alto, CA
pDsRed-Express-C1	Clontech	Palo Alto, CA
Kanamycine monosulfate	Sigma-Aldrich	Missouri, USA
TWEEN 20 Sigma Ultra	Sigma-Aldrich	Missouri, USA
Triton X-100 Sigma Ultra	Sigma-Aldrich	Missouri, USA
Polyvinyl alcohol 4-88	Fluka BioChemika	Buchs, Switzerland
Deoxynucleotide set 0.25ml of 100mM of dATP, dGTP, dCTP, dTTP	Sigma-Aldrich	Missouri, USA
Aqua Bidestillata	Mayrhofer Pharmazeutika GmbH	Leonding, Austria
Restriction enzymes	Promega	Madison, WI, USA
100 bp ladder	Promega	Madison, WI, USA
Generuler Low range DNA marker	Fermentas	St.Leon-Rot, Germany
Generuler Middle range DNA marker	Fermentas	St.Leon-Rot, Germany
1kB ladder	Promega	Madison, WI, USA
Restriction enzymes	Fermentas	St.Leon-Rot, Germany
Fast Digest restriction enzymes	Fermentas	St.Leon-Rot, Germany
Quantitas Fast DNA Marker	Biozym	Oldendorf, Germany
peqGOLD Protein-Marker V (Prestained)	PeqLab Biotechnologie GmbH	Erlangen, Germany
Ampicillin Sodium Salt	Sigma-Aldrich	Missouri, USA
4-Nitrphenylphosphate Disodium salt Hexahydrate >99% enzym	Fluka BioChemika	Buchs, Switzerland
LB Broth	Sigma-Aldrich	Missouri, USA
Agar	Sigma-Aldrich	Missouri, USA
24-well culture cluster	Corning Inc	NY, USA
Nitrocellulose membrane (pore size: 0,2µm)	PeqLab Biotechnologie GmbH	Erlangen, Germyn
Tgradient	Biometra	Goettingen, Germany

T3000 Thermocycler	Biometra	Goettingen, Germany
Glycerol anhydrous	Fluka, BioChemika	Buchs, Switzerland
Biozym LE Agarose	Biozym	Oldendorf, Germany
TOP10 E. coli	Invitrogen	California, USA
C2C12 cell line	DSMZ	Braunschweig, Germany
Coverslips ø 15mm	Menzel GmbH& Co KG	Braunschweig, Germany
Lumi-Light Western Blotting Substrate	Roche Diagnostics GmbH	Mannheim, Germany
Isopropanol	Sigma-Aldrich	Missouri, USA
Methanol	Sigma-Aldrich	Missouri, USA
Sodium dodecyl sulfate (SDS)	Fluka BioChemika	Buchs, Switzerland
TRIS(hydroxymethyl)aminomethane	Fluka BioChemika	Buchs, Switzerland
Sodium carbonate	Sigma-Aldrich	Missouri, USA
Ni-Sepharose gel affinity suspension	GE Healthcare	Amsterdam, the Netherlands
Rotilabo® syringe filter (0,22µm)	Roth	Karlsruhe, Germany
Complete EDTA-free Protease inhibitor cocktail	Roche Diagnostics GmBH	Mannheim, Germany
ECL solution: Luminol	Sigma-Aldrich	Missouri, USA
ECL solution: 4IPBA	Sigma-Aldrich	Missouri, USA
Lipfectamine 2000	Invitrogen	Lofer, Germany
Poly-L-Lysine (15,000-30,000 Da)	Sigma-Aldrich	Missouri, USA
SYBR green	Roche Diagnostics GmBH	Mannheim, Germany
Low-fat milk powder	Roth	Karlsruhe, Germany

Table 8: Chemicals used in this study

2.1.2. Equipment

Zeiss Axiovert 10	Carl Zeiss	Jena, Germany
Zeiss Axiovert 200M	Carl Zeiss	Jena, Germany
Tgradient	Biometra	Goettingen, Germany
T3000 Thermocycler	Biometra	Goettingen, Germany
Incubator CB150	Binder	Tuttlingen, Germany

Table 9: Equipment used in this study

2.1.3. Primers

The ordered primers are single stranded DNA-oligonucleotides purchased from Invitrogen (Lofer, Germany) or MWG-Biotech (Ebersberg, Germany). The annealing temperatures of the particular primers used were taken from the data sheet or calculated with an online primer calculator (Oligo Calculator: http://www.pitt.edu/~rsup/OligoCalc.html).

The ordered, lyophilized primers were diluted with ddH_2O to a final stock concentration of 100pmol/µl (final concentration of 10pmol/µl in a PCR reaction).

Primers for the amplification of Listeriolysin O (LLO)					
Name	**Sequence (5´to 3´)**	**T_A**	**Restriction site**	**Tag**	
LLO-s	GAATTCCATATGAAGGATGCATCTGCATTCAAT	61	NdeI		
LLO-as	GGGATCCTTATTATTCGATTGGATTATCTACT	59	BamHI		
LLO-His-as	GGATCCTTA**ATGATGATGATGATGATG**TTCGATTGGATTATCTACT	60	BamHI	His	
Primers used for creating the EYFP-His vector					
Name	**Sequence (5´to 3´)**	**T_A**	**Restriction site**	**Tag**	
EYFP-His s	GCCACCATGGTGAGCAAGGGCGAG	62	NcoI		
EYFP-His as	TTAATGATGATGATGATGATGCTTGTACAGCTCGTCCAT	62	EcoRI (backbone)		

Table 10: Primer used in this study

2.1.4. Vectors

Name	**Characteristics**	**Size (bp)**	**Manufacturer**
pCR2.1	TA cloning	3929	Invitrogen (Lofer, Germany)
pDsRed-Express-C1	Reporter vector for eukaryotic cells *Discosoma sp.* dsRed	4700	Clontech (Palo Alto, CA, USA)
pcDNA3	Overexpression in eukaryotic cells	5446	Invitrogen (Lofer, Germany)
pET11a	Bacterial expression vector	5677	Novagen (Madison, USA)
pCR2.1-LLO-His	TA cloning vector with Listeriolysin O	5400	This work
pET11a-LLO-His	Listeriolysin O expression vector	7180	This work

Table 10.1: Overview of vectors used in this study

2.1.4.1. pCR2.1

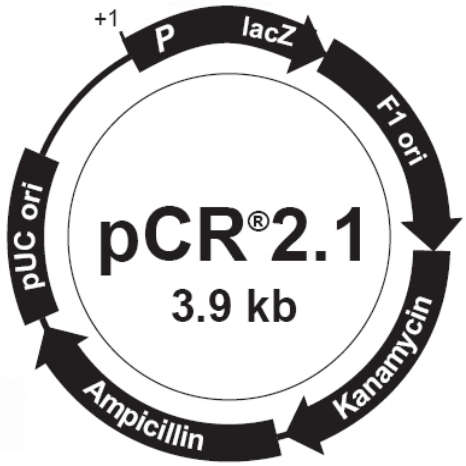


Figure 8: Vector map of pCR2.1: The linearized vector is equipped with an ampicillin and kanamycine resistance cassette, lacZα for blue white screening (α-complementation), T overhangs for direct TA insertion of amplified PCR product (section 2.2.4.2)

2.1.4.2. pDsRed-Express-C1

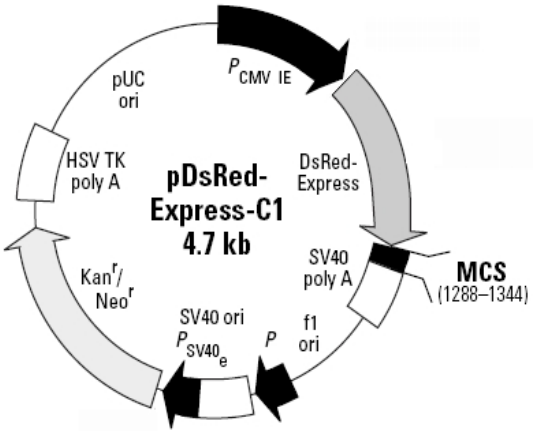


Figure 9: Vector map of pDsRed-Express-C1: the vector was used for overexpression of *Discosoma sp.* dsRed in eukaryotic cells under the SV40 CMV promoter. Optionally, due to the multiple cloning site located C-terminally to DsRed, an additional gene sequence can be inserted in order to create fusion proteins. For selection, the vector features a kanamycine and a neomycin resistance cassette.

2.1.4.3. pcDNA3

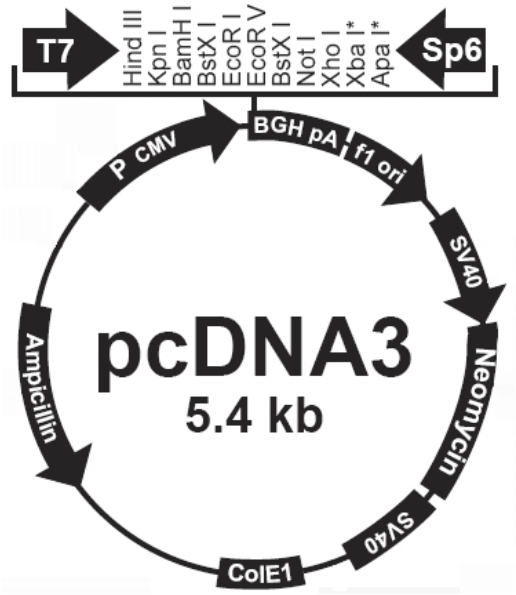


Figure 10: The vector map of pcDNA3 shows the multiple cloning site (MCS) and the upstream located SV40 CMV promoter region. An additional growth hormone polyadenylation signal (BGH) region allows the overexpression in eukaryotic cells. As selection markers, the vector features an ampicillin and a neomycin resistance cassette.

2.1.4.4. pET11a

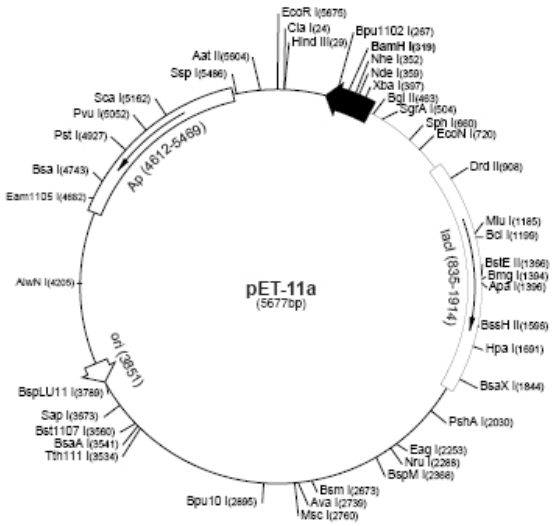


Figure 11: Vector map of pET11a depicting a multiple cloning site under an IPTG-inducible promoter for protein expression in prokaryotes.

2.1.4.5. pET11a-LLO-His

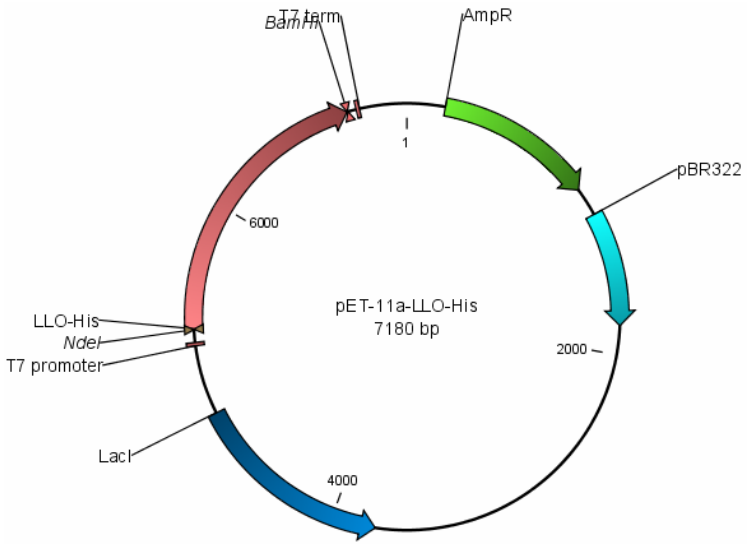


Figure 12: Vector map of pET11a-LLO-His: Listeriolysin O under control of an IPTG-inducible promoter for the protein expression in prokaryotes.

2.1.5. Bacterial strains

Genus/Species	Strain	Genotype	Manufacturer
Escherichia coli	TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen (Lofer, Germany)
Escherichia coli	BL21(DE3)	F–, *omp*T, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)	Invitrogen (Lofer, Germany)

Table 10.2: Bacterial strains used in this study

The TOP10 strain of *E. coli* was used for all cloning procedures, whereas the BL21 strain was exclusively applied for protein expression approaches.

2.1.6. DNA polymerases

Name	Characteristics	Manufacturer
Hot-Taq Polymerase	Standard polymerase	PeqLab (Erlangen, Germany)
Advantage-High Fidelity	Proofreading polymerase	Clontech (Palo Alto, CA, USA)

Table 10.3: Polymerases used in this study

2.1.7. Markers

2.1.7.1. DNA markers

DNA markers (separation by length) were diluted according to the manufacturer's instructions. Unless stated otherwise, 5µl of diluted DNA marker per slot was applied per agarose gel.

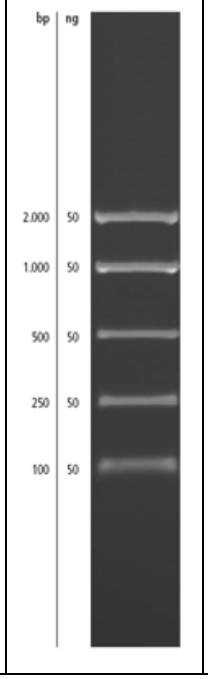
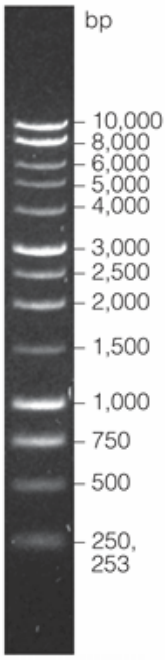
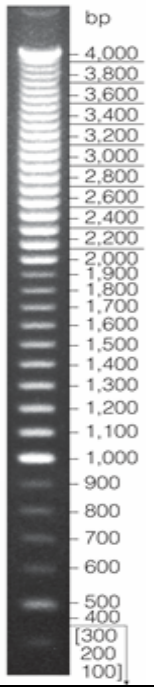
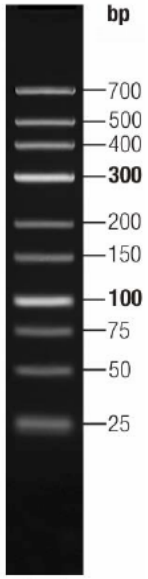
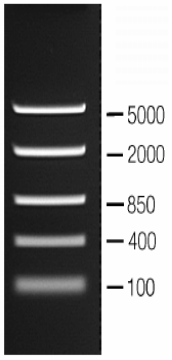
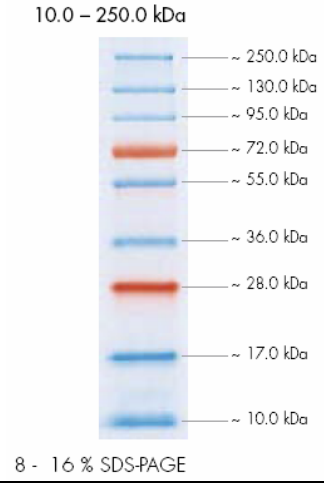
Name	Quantitas Fast 2kB	1kB benchtop ladder	100bp step ladder	GeneRuler™ DNA Ladder, Low Range	GeneRuler™ DNA Ladder, Middle Range
Min-Max (bp)	100-2000	250-10000	100-4000	25-700	100-5000
Company	Biozym (Oldendorf, Germany)	Promega (Madison, WI, USA)	Promega (Madison, WI, USA)	Fermentas	Fermentas
Ladder on agarose gel					

Table 10.4: DNA markers used in this study

2.1.7.2. Protein markers

Protein markers (separation by molecular weight) were diluted according to the manufacturer´s instructions. Unless stated otherwise, 5µl of diluted protein marker per PAGE gel was applied.

Name	peqGOLD Prestained Protein-Marker V
Min-Max (kDa)	10 - 250
Company	PeqLab Biotechnologie GmbH (Erlangen, Germany)
Ladder (on PAGE gel)	

2.1.8. Restriction enzymes

Restriction enzymes were used from Promega (Madison, WI, USA) and Fermentas (St.Leon-Rot, Germany) following the manufacturer´s instructions.

2.1.9. Antibodies

Monoclonal Anti-polyhistidine peroxidase conjugate	Sigma-Aldrich	Vienna, Austria
6X His tag® antibody (HRP)	Abcam	Cambridge, MA, USA

Table 10.5: Antibodies used in this study

2.1.10. Cell culture material

Dulbecco´s modified eagle medium (DMEM), high glucose	Sigma-Aldrich (Missouri, USA)
Fetal calves serum (FCS), heat inactivated	Lonza Ltd (Basel, Switzerland)
24-well plates (CoStar®)	Szabo-Scandic (Vienna, Austria)
6-well plates (CoStar®)	Szabo-Scandic (Vienna, Austria)
Cell culture flasks (T-25, T-175)	Greiner Bio One (Kremsmünster, Austria)
200mM L-Glutamine	Sigma-Aldrich (Missouri, USA)
Trypsin	Sigma-Aldrich (Missouri, USA)
10x Phosphate buffered saline (without calcium)	Lonza Ltd (Basel, Switzerland)

Table 10.6: Cell culture material used in this study

2.1.11. Cell line

Mouse skeletal myoblast precursor cell line (C2C12) (DSMZ#ACC565) was used for all cell cultural experimental procedures. For more information see section 2.4.1.

2.1.12. In silico analysis

Software and online sources used for cloning analysis (Table 10.7).

Vector NTI 9.0.0	C loning software	Invitrogen
NCBI	Gene and protein database, sequence BLAST,	http://www.ncbi.nlm.nih.gov/
Expasy	Verified protein database	http://www.expasy.org/
ClustalW2	Multiple alignments	http://www.ebi.ac.uk/Tools/clustalw2/index.html
Oligo Calculator	Calculation of the annealing temperatures of the primers	http://www.pitt.edu/~rsup/OligoCalc.html
CAP3	Sequence assembly program	http://pbil.univ-lyon1.fr/cap3.php
Chromas Lite	Chromatogram reader	http://www.technelysium.com.au/chromas_lite.html

Table 10.7: Software and online sources

2.2. Molecular Biology methods

2.2.1. Polymerase chain reaction (PCR)

Standard PCR mix	
1µl	Template DNA (5-20ng)

Primer-Stock (50µl)	
5µl	Primer sense (100pmol)

2µl	Primer stock
0.5µl	dNTPs
2.5µl	10x Puffer
0.3µl	Hot Taq-Polymerase
18.7µl	ddH_2O
25µl	

5µl	Primer antisense (100pmol)
40µl	ddH_2O

Table 11: Components for a standard mix (left table); 1:10 dilution of primers (right table)

Standard PCR program		
95°C	1min	
95°C	30sec	25-30 cycles
50°C	30sec	
X°C	30sec	
72°C	Ysec	
13°C	Pause	

PCR program for primers with overhangs		
95°C	1min	
95°C	30sec	8 cycles
50°C	30sec	
X°C	30sec	
72°C	Zsec	
95°C	30sec	25 cycles
Y°C	30sec	
72°C	Zsec	
13°C	pause	

Table 11.1: General PCR programs for the Taq polymerase (elongation temperature at 68°C instead of 72°C for the Advantage-HF proofreading polymerase)

Elongation time depends on the size of the PCR product, the annealing temperature on primer constitution and length.

General rule:

4x (number of G/C pairs) + 2x (number of A/T pairs) - 5

2.2.2. Agarose gel electrophoresis

All DNA separations by size were carried out on a 1% LE agarose gel (Biozym, Oldendorf, Germany) gel prepared in 1xTBE Buffer. The agarose gel/TBE buffer solution was heated in a microwave and cooled down to approximately 50°C following addition of a DNA chelating agent (Ethidium bromide (EtBr), GelStar® or GelRed®; final concentration of 0.3µg/ml) for detection. The DNA samples were mixed 6/1 in 6x gel loading buffer and loaded into the agarose gel (95-110V, 25-35min). Detection of DNA bands occurred under UV-light and photographed by a CCD-camera.

10X TBE buffer (1L)	**pH = 8.0**
108g	Tris
55g	Boric acid
40ml	0.5 M EDTA

Table 11.2: Composition of 10X TBE buffer for 1L

Gel loading buffer (6X)	
0.25%	Bromphenol blue (BPB)
0.25%	Xylene cyanol
30%	Glycerol
60.5%	ddH_2O

Table 11.3: Composition of 6x gel loading buffer

EtBr stock solution		**GelStar® stock solution**		**GelRed® solution**	
10mg/ml	in ddH_2O	10000x	in DMSO	10000x	in DMSO
Sigma-Aldrich, Missouri, USA		Cambrex, East Rutherford, NJ, USA		Biozym, Oldendorf, Germany	

Table 11.4: DNA chelating agents for the detection of DNA bands in agarose gels under UV-light

2.2.3. DNA extraction from agarose gels

Under UV illumination, the DNA band of interest was excised from the agarose gel with a scalpel. Then the gel piece was transferred into a 1.5ml Eppendorf tube and purified with the Promega Wizard SV Gel and PCR Clean-Up Kit (Madison, WI, USA) following the manufacturer´s instructions. As control, the purified samples were loaded on a 1% agarose gel.

2.2.4. DNA ligation

2.2.4.1. Conventional DNA ligation

Unless indicated otherwise, ligation of restricted DNA fragments with target vectors was carried out in a total volume of 10µl. Molar ratio of insert and vector DNA was estimated from the band intensities on an agarose gel. The following ligation mixture was incubated over night at 4°C.

Ligation		
Yµg	Target vector	**→ 4°C O/N**
3Yµg	Insert DNA	
Xµl	10x Buffer (Roche diagnostics)	
Xµl	T4 Ligase	
10Xµl	ddH_2O	
Total volume: 10Xµl		

Table 11.5: Components for DNA ligation

2.2.4.2. TA ligation/cloning

The TA cloning approach was carried out for direct ligation of amplified PCR products into the target vector. The pre-cut pCR2.1 features T-overhangs for insertion of PCR amplicons tagged with a terminal A overhang by the DNA polymerase.

TA-ligation

Yµg	precut pCR2.1	**→ 4°C O/N**
1-3Yµg	Insert DNA	
Xµl	10x Buffer (Roche diagnostics)	
Xµl	T4 Ligase	
10Xµl	ddH_2O	
Total volume: 10Xµl		

Table 11.6: Basic mixture of components for TA-ligation

2.2.5. *E. coli* culture (TOP10, BL21)

E. coli strains TOP10 and BL21(DE3) were handled identically according to the following culturing conditions. The bacterial cells from stored glycerol-stocks (or single colonies picked from the LB-agar plates) were inoculated in Luria Bertani Broth medium with the appropriate selective antibiotics (see table below) and grown overnight at 37°C under permanent shaking.

Antibiotics	**Shortcut**	**Properties**	**Working concentration**	**Stock concentration**
Ampicillin	Amp	kills dividing cells	50-100µg/ml	50mg/ml in ddH_2O
Chloramphenicol	Cm	bacteriostatic	20-170µg/ml	34mg/ml in Ethanol
Kanamycine	Kan	bactericidal	30µg/ml	50mg/ml in ddH_2O

Table 11.7: Properties and concentrations of antibiotics used in this study

Liquid LB medium	
10g	Tryptone
5g	Yeast extract
10g	NaCl
ddH_2O	1000ml

LB agar for plates	
10g	Tryptone
5g	Yeast extract
10g	NaCl
15g	Agar-Agar
ddH_2O	1000ml

Table 11.8: Media for bacterial cell culture

2.2.6. Preparation of competent *E. coli* (TOP10, BL21)

E. coli were grown in Luria Bertani Broth medium at 37°C under permanent shaking until an optical density of OD_{600} = 0.4-0.6 was reached . Afterwards, bacterial cells were chilled on ice for 10min following centrifugation at 4000rpm for 10min (4°C). After decanting of the supernatant, the pellet was resuspended in 30ml ice-cold Competent Buffer 1 (Table 11.9) and again centrifuged at the same conditions as in the step before. The supernatant was again removed and the pellet resuspended in 2ml ice-cold Competent Buffer 2 (Table 11.9). Finally, the competent cells were collected into 60 and 100µl aliquots, respectively, and shock-frozen in liquid nitrogen prior to the storage at -80°C.

Competent Buffer 1	
80mM	$MgCl_2$
20mM	$CaCl_2$
Resolved in ddH_20	

Competent Buffer 2	
100mM	$CaCl_2$
10%	Glycerol
Resolved in ddH_20	

Table 11.9: Buffers for the preparation of competent *E. coli*.

2.2.7. Transformation of bacterial cells

0.5-1µg plasmid DNA or 5µl of overnight ligation mix (section 2.2.4.1), respectively, were mixed with 20µl of thawed competent *E. coli* cells and chilled 30°C on ice. Heat-shock occurred at 42°C for 90sec followed by cooling down on ice for 2 minutes. After adding 80µl of SOC medium, solution was incubated at 37°C for 60 minutes under permanent shaking and plated on LB-agar plates containing the appropriate antibiotics for the selection of the transformants. For blue-white-selection (in case of the usage of pCR2.1), LB-agar plates were coated with X-Gal and IPTG (see Table 11.10 for the blue-white solution mix) prior to cell plating for the facilitated screening of positive clones.

Mix for blue white selection	
40µl	IPTG (100mM)
32µl	X-Gal (50mg/ml)
28µl	ddH_2O
→ 100µl per plate	

SOC medium	
2%	Tryptone
0.5%	Yeast extract
10mM	NaCl
2.5mM	KCl
10mM	$MgCl_2$
10mM	$MgSO_4$
20mM	Glucose

Table 11.10: Components for the transformation and selection of *E. coli*

2.2.8. Colony PCR for screening of positive clones

Screening of bacterial colonies for detection of positive clones with the desired DNA insertion in the plasmid was carried out by colony-PCR. Single bacterial colonies were picked and resuspended into 20µl liquid LB-medium. For the following PCR analysis, 1µl of the resuspended suspension was used for the PCR reaction. The PCR set-up is not changed, except for the elongated heating step at the first PCR step. Heating to 95°C for 5 minutes at the beginning of the PCR program ensures bursting of the bacterial cells making the hosted plasmid DNA accessible for polymerases and primers.

By choosing plasmid backbone primer flanking the region of the presumably inserted DNA, the size of the amplicon provide information about whether an insertion is existent or not (For pCR2.1: 150bp without insertion, 150bp + length of amplicon in case of a plasmid with inserted DNA fragment).

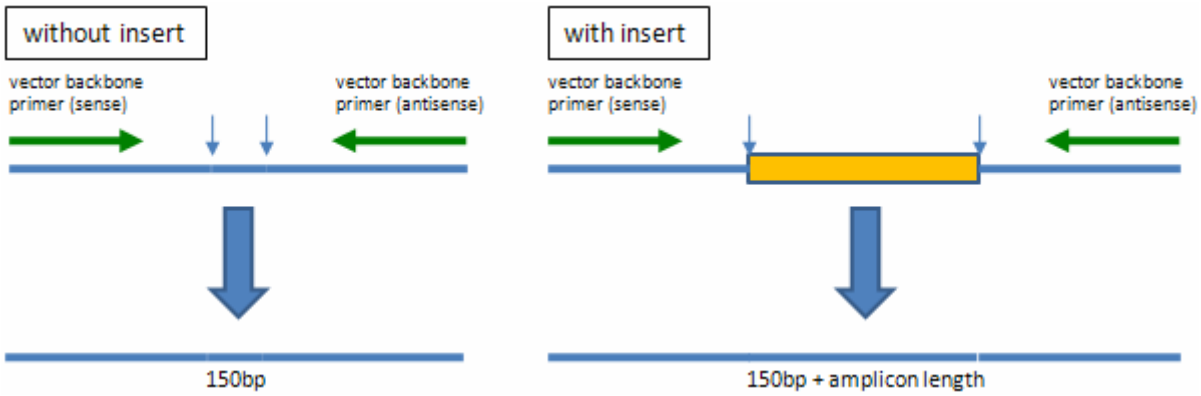


Figure 13: Schematic illustration of screening of positive clones by colony PCR

2.2.9. Plasmid isolation from E. coli (mini and maxi preparation)

Endotoxin-free plasmid isolation was carried out by the use of Promega Wizard® SV Minipreparation Kit (Promega, Madison, WI, USA) according to the manufacturer´s instructions. Plasmid yield was about 6-30µg (concentrations around 0.2-1µg/µl) depending on the copy-number of the plasmid per cell.

For larger DNA preparations, the Endo-free Maxiprep Kit, obtained from Qiagen (Hilden, Germany), was used to achieve DNA yields above 700µg (concentrations between 1 and 3µg/µl).

2.2.10. Quantification of DNA by photometry

The DNA concentration and purity was quantified by photometrical analysis using SmartSpec™ 3000, Biorad, Hercules (CA, USA). The DNA concentration was measured at the wavelength of A_{260}, whereas the absorption ratio A_{260}/A_{280} (DNA/protein) gave information about the purity of the isolated DNA.

2.2.11. Restriction of DNA

For appropriate conditions for the restriction enzymes, manufacturer´s instructions were followed concerning type and amount of buffer. Generally, incubation was performed for at least 2 hours at 37°C.

In the case of equal restriction ends of the target vector, the linearized vector was dephosphorylated at each 5´-end of the DNA strand in order to prevent religation prior to the insertion of introduced DNA during the ligation process.

DNA restriction	
1-5µg	1-5µg
0.5µl	Restriction enzyme
0.5µl	0.5µl
With ddH_2O to a total volume of 10µl	
Incubation at least for 2h at 37 °C	

Table 11.11: General mixture of a DNA restriction approach

Dephosphorylation	
2µg	linearized vector
2µl	10X Buffer
0.01unit/pmol of ends	Calf intestinal alkaline phosphatase (CIAP)
With ddH_2O to a total volume of 20µl	
Incubation for 30min at 37 °C	

Table 11.12: Standard dephosphorylation mix

2.2.12. DNA sequencing of plasmids

Sequencing of designed plasmids was carried out by using the sequencing service of MWG-Biotech (Ebersberg, Germany) or Microsynth (Balgach, Switzerland).

2.2.13. Design of the Listeriolysin O vector for bacterial expression

The *hlyA* gene from the *Listeria mononytogenes* strain DP-L2723 encoding LLO lacking its secretion signal sequence was amplified from the plasmid pDP3615 (a kind gift of Daniel Portnoy, University of California, Berkeley) using the sense primer LLO-s (5´-GAATTCCATATGAAGGATGCATCTGCATTCAAT-3´) generating an NdeI restriction site at the 5´end, and the antisense primer LLO-as (5´- GGGATCCTTATTATTCGATTGGATTATCTACT-3´) generating a BamHI restriction site at the 3´ end of the gene fragment. Additionally, for introducing a caboxy-terminal 6x-poly-histidinde tag, a second PCR reaction with the antisense primer LLO-His-as (5´-GGATCCTTAATGATGATGATGATGTTCGATTGGATTATCTACT-3´) was performed (for annealing temperatures and elongation times, Table 10).The amplicon was directly ligated into the pCR2.1 vector (section 2.2.4.2) and transformed into TOP10 *E. coli* cells (section 2.2.7) for storing, and into BL21(DE3) *E. coli* cells for protein expression, respectively. The recombinant LLO sequence was then transferred into the bacterial expression vector pET11a (NdeI, BamHI). BL21(DE3) cells where transformed with the vector ready for the protein expression approach (section 2.3.5).

2.3. Biochemical methods

2.3.1. Polyacrylamidegelelectrophoresis (PAGE) (non reducing)

During the migration in the electric field of the PAGE gel, the protein samples were separated according to their molecular weight. By the presence of sodium-dodecyl-sulfate (SDS), the proteins own surface charge is masked by gaining a negative net charge, making the migration velocity dependent on the protein size alone (the larger the protein, the more SDS can bind (increasing of surface negative charge).

Protein samples were denatured at 95°C in a thermo-block for 5min and loaded onto a 12% PAGE gel using capillary tips. Prior to applying a higher voltage, a prerun with 60 Volts for 20 minutes ensured a better compression and resolution of the protein bands. The subsequent electric field of 150-160 Volts was sustained until a sufficient separation of the prestained protein marker was achieved.

4X Separating Buffer	
17.9g	Tris
3.8g	Tris-HCl
0.4g	SDS
With ddH_2O to 100ml	

4X Stacking Buffer	
0.3g	Tris
7.5g	Tris-HCl
0.4g	SDS
With ddH_2O to 100ml	

5X Running Buffer	
15g	Tris
72g	Glycine
5g	SDS
With ddH_2O to 1000ml	

Table 12: Components for PAGE buffer

Separating gel (12%)	
2.4ml	4X Separating Buffer
3.4ml	ddH_2O
4ml	30% AA/Bis-AA
100µl	10% APS
4µl	TEMED

Stacking gel	
0.75ml	4X Stacking Buffer
1.8ml	ddH_2O
0.45ml	30% AA/Bis-AA
30µl	10% APS
3µl	TEMED

Table 12.1: Mixture of PAGE buffers and additional components for gelation

2.3.2. Non specific protein staining (Coomassie dye)

Non-specific labeling of protein bands was conducted with Coomassie Brilliant Blue R-250. The PAGE gel was incubated in staining buffer for 30min following three steps in destaining buffer to wash out the dye from areas of the PAGE gel without any protein deposit. Each destaining step was carried out for 20min following renewal of destaining buffer. Destained gels were incubated over night in ddH_2O.

Staining buffer	
450ml	Methanol
450ml	ddH_2O
100ml	Acetic acid
2,5g	Coomassie Blue R-250

Destaining buffer	
450ml	Methanol
450ml	ddH_2O
100ml	Acetic acid

Table 12.2: Buffer components for non specific protein staining

2.3.3. Semi-dry western blot

Western blot for specific protein labeling was carried out using antibodies recognizing the c-terminally poly histidine epitope.

2.3.3.1. Protein transfer to nitrocellulose membrane

The PAGE gel, the nitrocellulose (NC) membrane (BioRad, Hercules, CA, USA) and the filters were equilibrated in 1X blotting buffer for 15min at room temperature. The transfer of the protein bands to NC was carried out for 40min under a constant electric field of 10V (for schematic illustration see Figure 14). Prior to the antibody staining, blotted NC membrane was incubated with the reversible protein dye ponceau red for 15 minutes at room temperature to verify the efficient transfer of the protein bands to the membrane. After verification, ponceau red was washed out with 1X PBS.

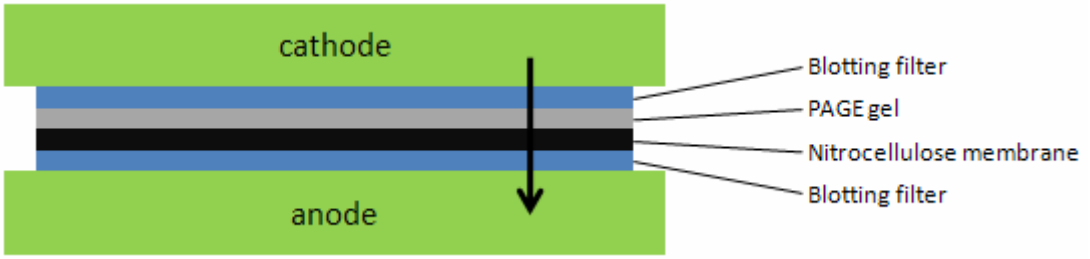


Figure 14: Schematic illustration of the blotting apparatus

2.3.3.2. Single antibody staining

Prior to the antibody addition, the NC membrane was blocked with 5% low-fat milk purchased from Roth (Karlsruhe, Germany) or with 2% bovine serum albumin in order to reduce non-specific bindings of the antibody on the NC membrane. The blocking reaction was conducted either for 2 hours at room temperature or over night at 4°C. Subsequent washing of the NC membrane was carried out with 1X PBST for 5 minutes in order to remove all non-bounded antibodies. The 6x His HRP-conjugated antibody was diluted 1:2000 in 1X PBST in the presence of 0.25% low-fat milk and added to the NC membrane for 1 hour in the dark at room temperature (for antibody information see Table 10.5). After washing of the membrane three times with 10ml 1X PBST the membrane was drained and ready for detection.

10X blotting buffer	
48mM	Tris
39mM	Glycine
0,037%	SDS

1X blotting buffer	
140ml	ddH_2O
20ml	10X blotting buffer
40ml	Methanol

1X PBS (pH = 7.5)	
0.2g	KH_2PO_4
1.44g	$Na_2HPO_4x2\ H_2O$
8.18g	NaCl
0.2g	KCl
1L	ddH_2O

Blocking solution
2% BSA in 1xPBS

PBST
0.2% TWEEN in 1X PBS

Table 12.3: Buffer components for the Western blot

2.3.3.3. Detection of antibodies

The antibody was detected by the enzymatic activity of the conjugated horseradish peroxidase. As ligand for luminescence detection, the blocked NC membrane was incubated for 1min with the ECL solution (Sigma-Aldrich) prior detection.

Luminol stock	
250mM Luminol	44.3mg in 1ml DMSO

4-iodophenylboronic acid (4IPBA) stock	
90mM	111.5mg in 5ml DMSO
ECL buffer	
100mM	Tris-HCl (pH = 8.8)

Table: Stock concentrations and buffer for the ECL solution

Working dilutions	
1,25mM	Luminol (1:200)
2mM	4IPBA (1:45)
5,3mM	H_2O_2 (1:166 from 3% stock

Table 12.4: Working dilutions of ECL solution

For 5ml ECL solution	
25µl	Luminol
111µl	4IPBA
30µl	H_2O_2
Incubation on NC membrane: 1min	

Table 12.5: mix for 5ml ECL solution

2.3.4. DNA condensation kinetics with PLL

Plasmid and linear DNA, respectively, were incubated with the DNA fluorescence dye SYBR green and different concentrations of the polycation poly-L-lysine (15.30 kDa). The kinetic of the formation of the DNA/polycation microparticles was observed under fluorescence microscopy.

2.3.5. Expression of LLO in E. coli

E. coli cells (BL21) housing the LLO expression vector pET11a-LLO-His (section 2.1.4.5, Figure 12) were inoculated in 5ml LB medium with the appropriate antibiotics for selection and grown over night at 37°C under shaking conditions. The following day, the overnight culture was diluted with 400ml LB medium and growth was proceeded until the absorbance at the wavelength of 600nm reached the threshold of 0.4-0.6 (still in the exponential growth phase). Due to the IPTG-inducible promoter located upstream of the protein sequence of the pET11a vector, the subsequent induction of the protein expression was launched by the addition of IPTG (0.3mM final concentration). After the induction, the bacterial suspension was incubated either at room temperature or at 37°C, and aliquots after defined time points (1h – 16h) were collected to obtain optimal expression conditions. For LLO, the incubation period of 4 hours showed an optimal protein yield to cell mass ratio.

After the protein expression, the bacterial suspensions were aliquoted into 50ml tubes and the cells were harvested by centrifugation with 4000rpm at 4°C for 30min. Subsequent washing of the pellets with 1X PBS followed by an additional centrifugation step ensured the elimination of remaining medium components. The washed cell pellets were stored at -80°C for the subsequent protein purification procedure.

2.3.6. Purification of LLO

2.3.6.1. Lysis of E. coli BL21

The frozen pellet of 50ml bacterial over night culture was thawed on ice. The lysis of *E. coli* was carried out using 2ml of Lysis buffer (see table at the end of this section). After incubation at room temperature for 30min under permanent shaking, the cell suspension was sonicated three times for 15 seconds on ice in order to degrade genomic DNA and lyse remaining cells. Cell debris and inclusion bodies were spinned down for 20min at 4000rpm (4°C).

2.3.6.2. Washing of LLO inclusion bodies

The washing of the inclusion bodies was performed two times with 20ml of washing buffer blank following sonication and centrifugation at the same conditions as above. Unless otherwise indicated, additionally a third washing step with washing buffer triton was carried out.

2.3.6.3. Solubilization of LLO in inclusion bodies

For solubilizing proteins in the inclusion bodies, the pellet was resuspended in 5-10ml Solubilization buffer following sonication and incubation at least for 20min at room temperature.

2.3.6.4. Refolding of LLO

For renaturation, the solution was diluted in 1X PBS, 1X PBS with 500mM NaCl, or acidic buffer at pH = 5 (Table 12.8). Optionally, for protein stability analysis, the protein was diluted 1:10 in refolding buffer.

2.3.6.5. Purification of recombinant LLO by affinity gel chromatography


Figure 15: Self-made affinity column

For purification of the recombinant LLO (C-terminal 6xHis-Tag) by affinity chromatography, a 3ml syringe attached to a Rotilabo® syringe filter (0.22µm) purchased from Roth (Karlsruhe, Germany) was used as container for the Ni-Sepharose gel affinity suspension (GE Healthcare, Amsterdam, the Netherlands). The refolded protein suspension (or form purification step directly after the solubilization with guanidine hydrochloride (Gnd-HCl)) was diluted 1:1 with binding buffer and loaded onto the column pre-equilibrated with 3 column volumes of equilibration buffer (9ml). The elution of LLO was performed with 1 column volume of elution buffer. Optionally, the elution buffer was pushed through the column by the syringe plunger.

Lysis buffer (for 2ml 1X PBS)	
2mg/ml	Lysozyme
½ Tablet	Complete EDTA-free protease Inhibitor (Roche diagnostics)
50µg	Dnase I

Washing buffer blank	
100mM	Tris (Base)
1mM	(EDTA)
pH = 8	

Washing buffer triton	
100mM	Tris (Base)
1mM	(EDTA)
2%	Triton X100
pH = 8	

Solubilization buffer	
4(-8M)	Gnd-HCl
100mM	Tris (Base)
500mM	NaCl
EDTA-free (Ni-NTA column!)	
Otherwise 2mM EDTA	

Refolding buffer	
50mM	Tris (Base)
0,7M	Arginine (or CHES)
1M	NaCl
0,3M	Gnd-HCl
pH = 8,5	

Table 12.6: Buffers for purification of the recombinant protein listeriolysin O (LLO)

Binding buffer	
20mM	Phosphate buffer
500mM	NaCl
20mM	Imidazole

Elution buffer	
20mM	Phosphate buffer
500mM	NaCl
500mM	Imidazole

2.3.6.6. Dialysis of LLO

In order to remove the leftovers of the solubilization buffer, the refolded LLO (by dilution) was dialyzed in either PBS (pH = 7.4) or acidic buffer (pH = 5), respectively. The dialysis was performed in two overnight steps. At first step, 25µg/µl LLO (total volume of 2ml) was incubated over night in 50ml buffer at 4°C, following replacement of the buffer with further 50ml of buffer, incubating a second time at same conditions as above.

2.3.7. Determination of protein concentration

For protein quantification, a colorimetric method using the Bradford reagent was performed. After non-specific binding of the Coomassie Brilliant Blue G-250 dye to proteins, a shift in the absorption maximum from 470nm to 595nm dependent on the protein amount can be detected allowing a sensitive calculation of the protein amount. For unknown proteins like LLO, a calibration line (absorption units/concentration) of bovine serum albumin (BSA) was established as a benchmark in order to conclude the concentration of purified protein from the absorption value (Figure 16).

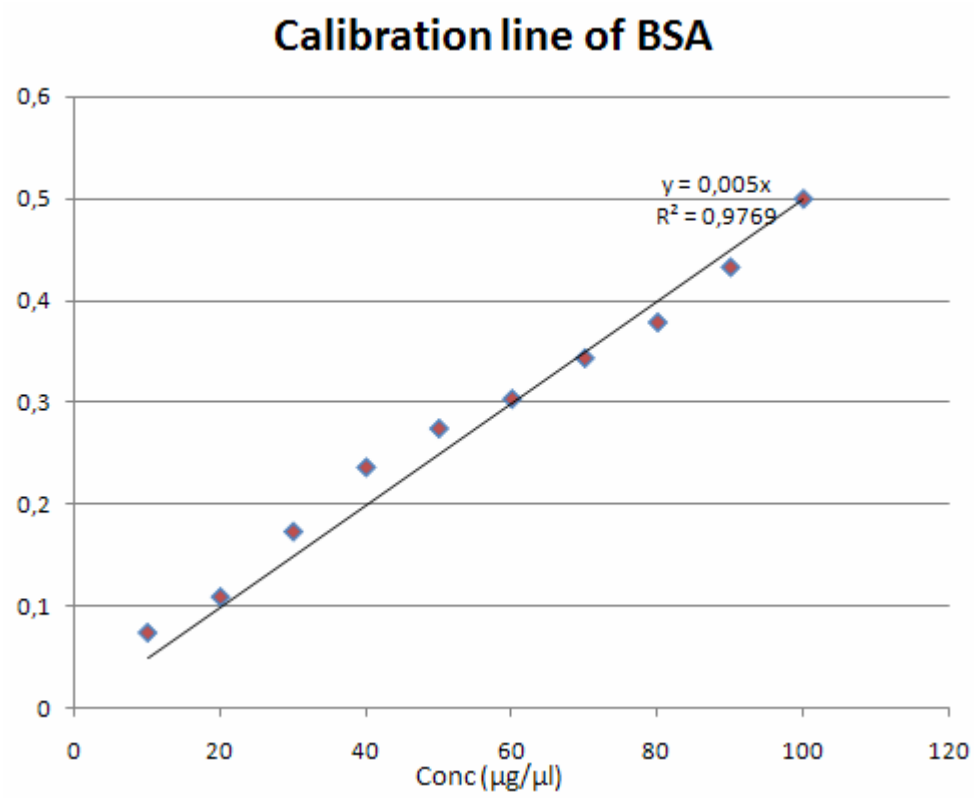


Figure 16: Calibration line of bovine serum albumin

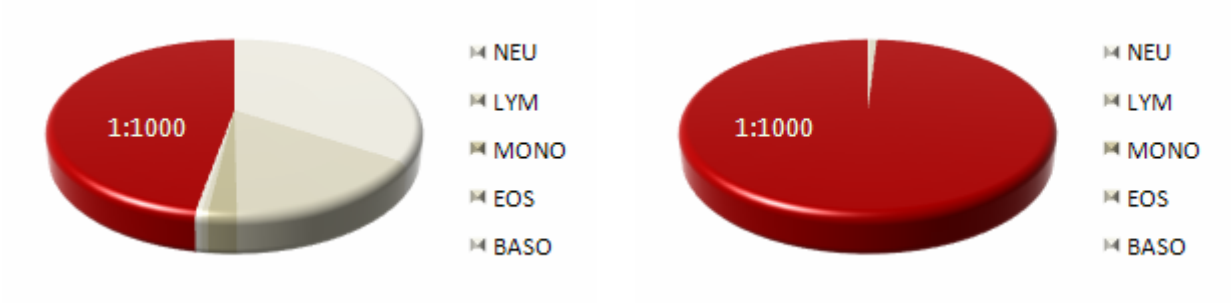
2.3.8. Hemolytic assay

To quantify the membrane disrupting activity of a protein, washed red blood cells (RBCs) were applied as ligands.

2.3.8.1. Washing of red blood cells

6ml of human blood was collected, the serum supernatant was removed and the pellet containing the pure red blood cells was washed with 20ml of the iso-osmotic buffer 1X PBS following centrifugation at 1800rpm for 20min at 4°C. The washing step was repeated twice to remove white blood cells located on the intermediate phase and remaining serum components. Purity of washed red blood cells was determined by FACS analysis.

Before washing **After washing**



WBC	NEU	LYM	MONO	EOS	BASO	RBC	K/µl	WBC	NEU	LYM	MONO	EOS	BASO	RBC
5,62	3,65	1,65	0,22	0,05	0,0048	5000		0,04	0,01	0,03	0	0	0	4200

Figure 17: Washing of red blood cells (NEU = neutrophils; LYM = lymphocytes; MONO = monocytes; EOS = eosinophiles; BASO = basophils)

2.3.8.2. Determination of the activity of the hemolytic protein LLO

Washed RBCs were diluted 1:10 (450µl) with solutions dependent on the approach indicated in Table 12.7. The amount of applied hemolytic protein and incubation time and temperature are indicated in the description.

Approach	**Solution for dilution used**
Hemolytic assay (pH=7)	1xPBS
Hemolytic assay (pH=5)	Acidic buffer
Positive control	ddH_2O
Negative control	1X PBS
pH gradient	Phosphate buffer gradient (pH = 4-7.6)

Table 12.7: Applied buffers for the dilution of red blood cells to ensure different pH and osmotic conditions for hemolytic proteins

Diluted RBCs were incubated in the indicated temperature, pH and time conditions.

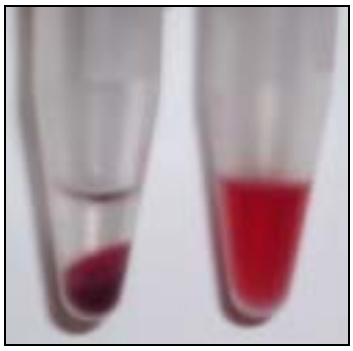


Figure 18: Centrifuged non-lysed (left) and lysed (right) RBCs

After the incubation period, the RBCs were centrifuged at maximum speed (13000rpm) for 5 minutes. The hemoglobin molecules of non-lysed RBCs remained inside the cell and were spinned down due to the larger weight compared to lysed RBCs without encapsulated hemoglobin. The supernatant was carefully transferred into a new tube and diluted 1:20 with the adequate buffer for the subsequent photometric quantification. The released hemoglobin is photometrically detectable at the wavelength of 541nm and gave information about the amount of lysed RBC. Additionally, to give a relative benchmark for the activity of hemolytic proteins, RBCs were lysed by the hypo-osmotic force in presence of ddH_2O. As negative control, RBCs were diluted in 1X PBS alone (without hemolytic protein).

2.3.9. LLO stability assay

For stability tests, purified LLO was stored in different pH, salt and temperature conditions, respectively (Table 12.8). At different time points, protein aliquots were tested for their hemolytic activity in red blood cells.

Concentration of LLO (ng/µl)	Time points (hours)	pH	Temperature
20-100µg/ml	0-2 weeks	5-7	4°C, RT

Table 12.8: Different tested storage conditions for LLO

2.4. Cell biology methods

2.4.1. Culture of C2C12 mouse myoblast cell line

The mouse myocyte precursor cell line (DSMZ#ACC565) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cell line was incubated at 37°C (5% CO_2) in medium indicated in the table below. Passaging of cells was carried out in order to obtain a maximal confluency of approximately 80-90% (a higher cell density leads to myogenic differentiation of the cells).

1% (2mM)	L-glutamine
5%	Fetal calf serum (FCS)
In DMEM high glucose	

Table 12.9: Medium components for the culture of C2C12 cells

2.4.2. Storage of C2C12

One million of harbored cells (DMEM with 5% FCS) were mixed with DMSO (final concentration of 10% v/v) and frozen in liquid nitrogen.

2.4.3. Lipofection of C2C12

For *in vitro* lipofection of C2C12 cells with endotoxin-free plasmid DNA, liposomal transfection reagent (Lipofectamine 2000) was obtained from Invitrogen (Lofer, Germany). By providing a net positive charge, the cationic liposomal particles interact ionically with the negatively charged DNA backbone forming microparticles. The complexed DNA/cationic liposome particles are endocytosed by the target cells. In order to avoid cross reactions of the cationic liposomes with serum components, the complexation reaction with the endotoxin-free DNA was carried out in serum-free medium. Unless otherwise indicated, a general molecular ratio of 1:1 (µg DNA : µl Lipofectamine 2000) with maximal amounts of 2µg DNA was applied per well in a 24-well plate for this studies. After incubation at 37°C (5% CO_2) for 4 hours, the serum-free medium was replaced by DMEM high glucose containing 5%

FCS to prevent cytotoxic effects of cationic liposomes. For positive control, the transfection efficiency was tested using the fluorescence reporter vector pcDNA3-EYFP-His, encoding for the enhanced yellow fluorescence protein (EYFP). The expressed reporter protein is detectable after 12-18 hours following transfection by fluorescence microscopy.

2.4.4. Transfection of C2C12 with PLL

C2C12 cells were grown at 37°C (5% CO_2) to a confluence of 80% in DMEM high glucose with 1% L-glutamine and 5% FCS. The pCDNA-EYFPHis vector was complexed with PLL at different molar ratios and incubated in 100µl serum-free DMEM high glucose for 15 minutes following transfer to the cells (24-well plate). Detection by fluorescence microscopy was carried out 24 hours, 48 hours and 96 hours post-transfection, respectively.

2.4.5. DNA condensation analysis with PLL

2.4.5.1. Electromobility shift assay (EMSA)

EMSA was used to detect DNA binding substances like PLL. The applied positively charged PLLs were able to bind to the negatively charged DNA backbone via electrostatic interactions. To show binding kinetics, double-stranded circular DNA at different sizes was incubated with PLL (15-30kDa) at different molar ratios. By showing differences in the migration velocity depending on the amount of complexation, the rate of condensation can be relatively evaluated. Non-bound DNA shows the highest migration velocity.

2.4.5.2. Condensation kinetics with fluorescence microscopy

DNA complexation velocities were demonstrated with fluorescence microscopy by the usage of circular and linear DNA pre-labeled with equimolar amounts of SYBR green. After the addition of PLL to the labeled DNA (molar ratio of 1:30), fluorescence images were captured every 10 seconds.

2.4.6. Uptake kinetics of PLL condensed DNA (and SYBR green) with and without LLO

For observing the uptake frequency of DNA/PLL particles, DNA was pre-labeled with SYBR green (Roche Diagnostics GmbH) in order to localize the microparticles under fluorescence illumination. The fluorescence dye is binding to double-stranded DNA molecules and emitting light after excitation with fluorescence light (excitation at 494nm, emission at 521nm). The resulting labeled plasmid DNA was complexed with PLL at weight ratios ranging from 1:10 to 1:30 for 10 minutes in 100µl of serum free medium or 1xPBS. Additionally, as presumable transfection enhancer, LLO was pre-incubated with PLL for 10 minutes prior to the addition of labeled DNA. Complexed particles with and without LLO were then transferred to 24-well plates containing C2C12 cells with densities of up to 90% per well. C2C12 cells were incubated for 15 minutes at 37°C (5% CO_2) followed by detection under fluorescence light.

2.4.7. Immunohistochemistry

Cellular localization of uptaken LLO was confirmed by direct immunohistochemistry. Additionally, for testing the epitope specificity of the Anti-His antibody, a control staining of cells transfected with pcDNA3-EYFP-His was additionally carried out.

C2C12 cells were grown on poly-L-lysine coated coverslips (VWR International, West Chester, PA, USA) in 24-well plates for subsequent detection under confocal laser scanning microscopy (CLSM).

In a laminar flow sterile workbench, coverslips (0.6cm) were washed with 70% ethanol following an autoclave step for sterilization. Coverslips were incubated 1 hour at room temperature in the poly-L-Lysine solution (Sigma-Aldrich) prediluted 1:10 with ddH_2O. Coverslips were washed twice to remove additional non-bound poly-L-Lysine and air-dried under UV for 30min. Coverslips were stored at 4°C maximal for 6 weeks. To maintain the fluorescence activity of GFP, EYFP and DsRed, the conservation of the native conformation was achieved by adopting a cell fixation protocol using methanol and formaldehyde. C2C12 cells grown on coverslips (24-well plate) were washed once with 2ml 1X PBS following fixation with 1ml 2% formaldehyde per well for 15minutes at 37°C. Then, wells were washed three times with 1X PBS. For permeabilization of the cell membrane, each well was incubated for 5minutes with 500µl methanol (-20°C) on a cooled metal block (-20°C). followed by three additional washing steps with 1X PBS to remove the remaining methanol. Blocking with 1ml 5% BSA in 1xPBS for 1 hour at room temperature was performed in order to prevent non-specific binding of the antibodies and therefore reduce the background signal. The antibody was diluted in 200µl 1xPBS (Table 10.5) and added to the fixed cells. The incubation in the dark was performed for 1 hour. Coverslips were washed three times for 5 minutes with 1xPBS and mounted onto superfrost slides. For a 100ml mowiol 4-88 mounting solution, 12g Glycerol were added to 4.8g mowiol 4-88 and continuously stirred whilst maintaining a constant temperature of 50°C in a water bath. The solution was diluted by the addition of 12ml ddH_2O following 24ml of 0.2M Tris-HCl and continuing mixing and heating until the mowiol was fully dissolved. The solution was then centrifuged at 8000rpm for 15min in order to achieve a clear solution. 100µl aliquots for the later usage were stored at -20°C. 7-10µl of the mowiol solution was prepared in drops on superfrost slides for each coverslip. Coverslips with fixed cells were removed from the 24-well plate and mounted upside down onto the mowiol drop. The slides were dried overnight at room temperature for subsequent analysis by CLSM.

3. Results

3.1. Cloning of recombinant Listeriolysin O

The LLO-His fragment restricted from pCR2.1-LLO-His with NdeI and BamHI prior the insertion into a bacterial expression is shown in Figure 19. The schematic illustration of the subsequent restriction fragment is depicted in Figure 20.

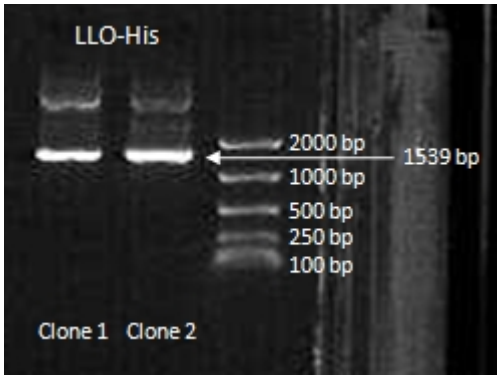


Figure 19: LLO-His fragment restricted from pCR2.1-LLO-His on a 1% agarose gel. Two restriction digests from starting vectors derived from different *E. coli* clones (clone 1 and clone 2).

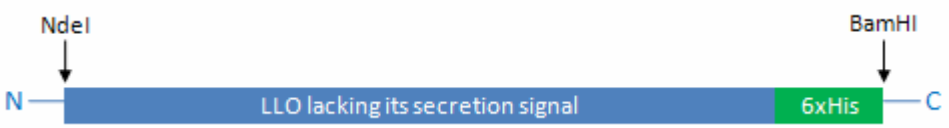
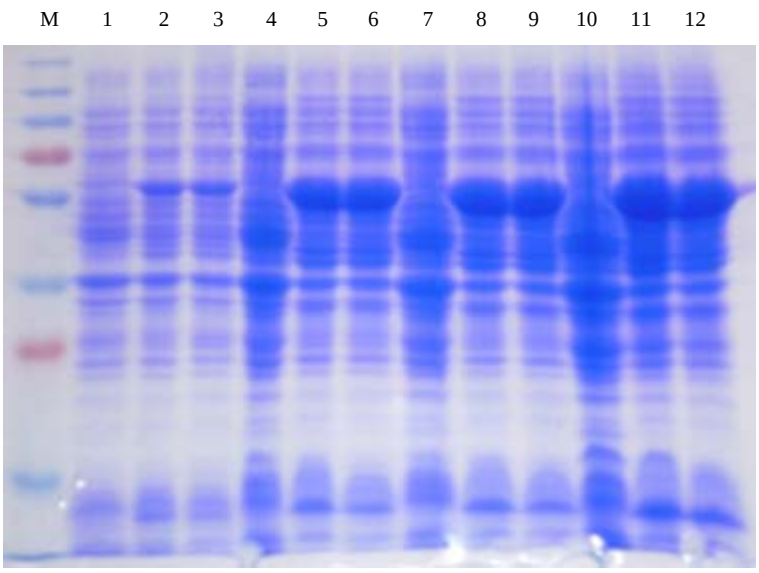


Figure 20: Schematic illustration of LLO amplicon lacking the secretion signal and containing of a C-terminal His-tag.

3.2. Expression of LLO in *E. coli* BL21(DE3)

For the optimization of the expression of the recombinant LLO, a screen with different expression conditions was performed (Figure 21). First, the influence of the applied IPTG concentration was determined and revealed no correlation of protein amount and IPTG concentration. Additionally, it was observed that an induction time of 4 hours was sufficient for an optimal protein yield.



Lane	IPTG conc. (mM)	Induction time (hours)
M		
1	0	1
2	0.3	
3	10	
4	0	2
5	0.3	
6	10	
7	0	3
8	0.3	
9	10	
10	0	16 (over night)
11	0.3	
12	10	

Figure 21: PAGE gel of protein expression screen with altered IPTG concentration and induction time, respectively. After the induction time, *E. coli* BL21(DE3) cells were directly heated to 95°C and mixed with 1 volume of 2X SDS loading buffer and subsequently loaded onto the PAGE gel (10%, 150V, 1 hour). PeqGold Protein Marker V was used as protein ladder (section 2.1.7.2).

The results from additional expression screens (Figure 22) revealed that the protein remained in the pellet fraction forming inclusion bodies. Additionally, no significant difference in the protein yield or degradation was observed by protein expression at room temperature or at 37°C, respectively.

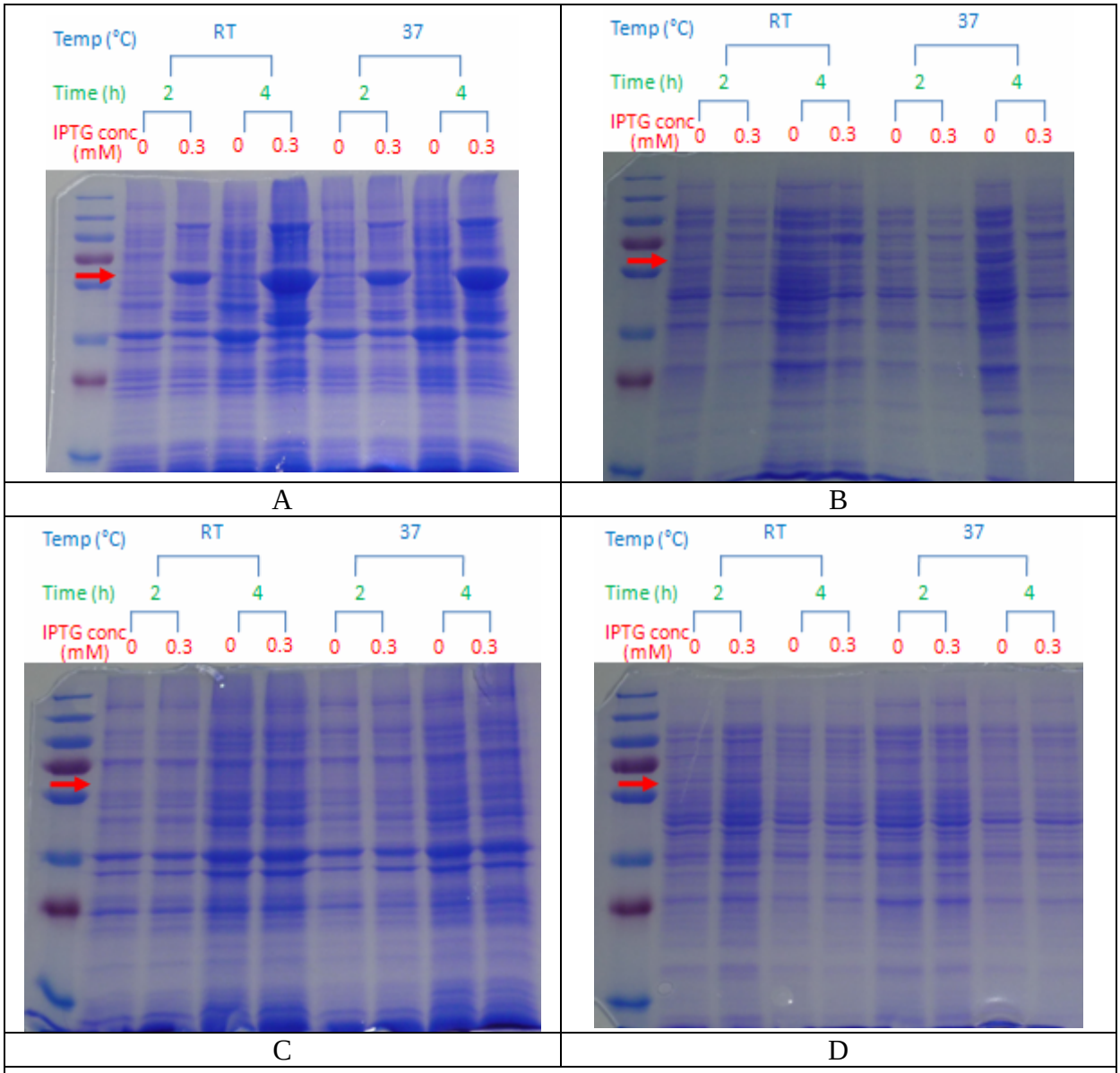


Figure 22: PAGE gels performed after the protein expression at different conditions (RT, 37°C, 2h, 4h, 0mM IPTG, 0,3mM IPTG) (A-D). Pellet fraction of the induction of the bacterial expression vector pET11a-LLO-His in BL21(DE3) cells at different conditions (A), supernatant fraction of the induction of the bacterial expression vector pET11a-LLO-His in BL21(DE3) cells at different conditions (B), Pellet fraction of the induction of the bacterial expression vector pET11a in BL21(DE3) cells at different conditions as control (C), supernatant fraction of the induction of the bacterial expression vector pET11a in BL21(DE3) cells at different conditions as control (D). The red arrow shows the excepted band height of LLO. RT = room temperature, IPTG = final concentration of applied IPTG (mM).

The expressed LLO was qualitatively verified by Western blot using antibodies recognizing a previously attached C-terminal His-tag domain to LLO (Figure 23), showing additional several degradation products of LLO. For further purification, Ni-NTA affinity chromatography was

performed and 10µl of the eluted fraction was loaded onto a PAGE gel for verification (Figure 24).

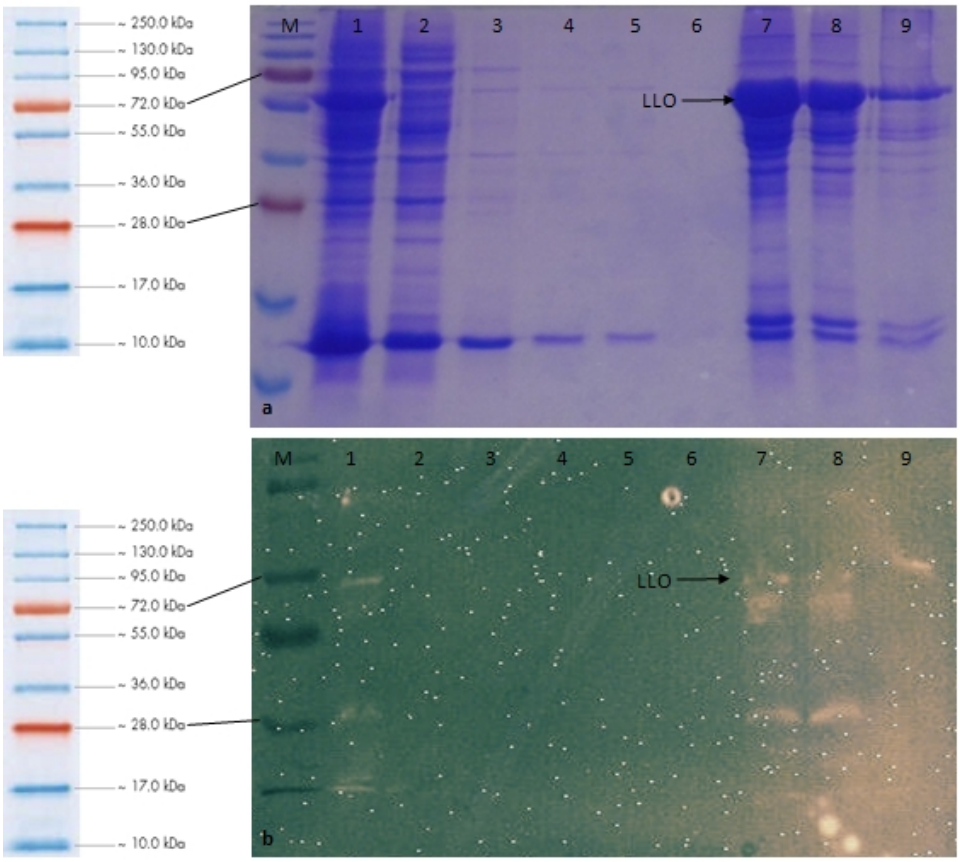


Figure 23: PAGE gel (a) and Western blot (b) of the expression and purification steps of Listeriolysin O (LLO) (10%). PeqGold Protein Marker (M), proteome of lysed *E. coli* cells expressing LLO (1), soluble protein fraction of LLO expressing *E. coli* cells after centrifugation (20min, 4000rpm) (2), first washing step with 20ml PBS (3), second washing step with 20ml PBS (4), third washing step with 20ml PBS (5), forth washing step with 20ml PBS (6), remaining pellet of inclusion bodies after the washing steps (7), 1:1 dilution of the remaining pellet of inclusion bodies after the washing steps (8), dialysis (over night at 4°C) of LLO in acidic buffer (pH = 5) (9). Staining of Western blot was performed with anti-His antibodies conjugated with horseradish peroxidase.

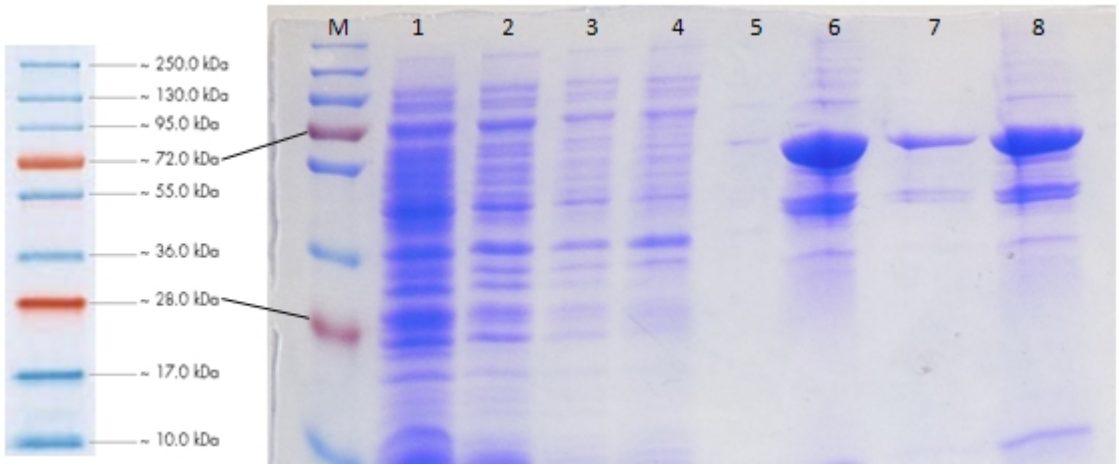


Figure 24: PAGE gel of the expression and purification steps of Listeriolysin O (LLO) (10%). PeqGold Protein Marker (M), soluble protein fraction of LLO expressing *E. coli* cells after centrifugation (20min, 4000rpm) (1), first washing step with 20ml PBS (2), second washing step with 20ml PBS (3), third washing step with 20ml PBS (4), forth washing step with 20ml PBS (5), remaining pellet of inclusion bodies after the washing steps (6), inclusion bodies solubilized in 5ml 4M Guanidine-hydrochloride (diluted 1:20 with PBS for the PAGE gel) (7), LLO fraction after purification with affinity chromatography (His-tag) (8),

From a bacterial starting solution of 50ml, a yield 450µg/ml LLO was achieved (total amount of 8mg) (AU of purified LLO in 20ml buffer following dilution 1:10 in PBS: 0,203). Table 13 shows currently achieved protein yields of expression of recombinant LLO in *E. coli*.

Culture volume of *E. coli* (ml)	Yield		Procedures	Ref.
	mg/L	Total (mg)		
1000	4.5	4.5	hydroxyapatite adsorption chromatography, ammonium sulfate precipitation, dialysis, Ion-exchange chromatography, ultrafiltration for concentration	[312]
600	2.5	1.5	immobilized metal affinity chromatography	[313]
50	40	8	His-tag affinity chromatography	This work
600	40	> 90		

Table 13: Currently achieved protein yields of expression of recombinant LLO in *E. coli*

The yield of total amount of LLO was increased dramatically.

3.3. pH dependent hemolytic activity of LLO

The pH dependent hemolytic activity of purified LLO (Figure 25). LLO showed maximum capability to lyse red blood cells at pH = 5.5. The hemolytic activity was dramatically decreased upon the increase of pH above 6.

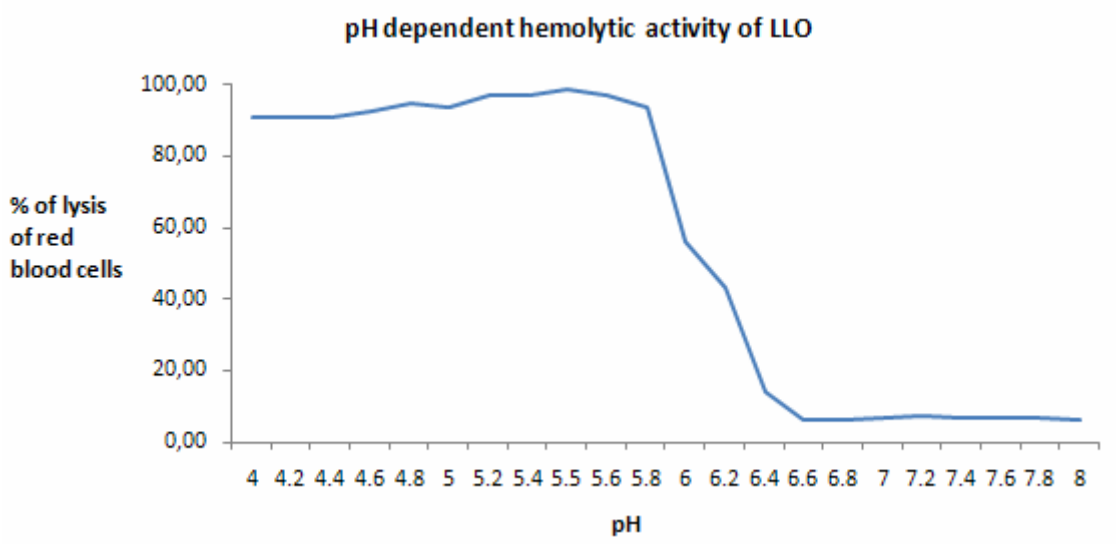


Figure 25: pH dependent hemolytic activity of purified LLO (percentage relative to 100% lysis of red blood cells with ddH_2O). 100ng of LLO were incubated for 5min at 37°C and centrifuged for 5min at 13.000rpm. The absorption at 541nm was measured photometrically.

3.4. LLO stability assay

40-100ng of LLO purified with slightly different procedures was incubated in washed and prediluted RBCs. By solubilization of LLO with different volumes of the solubilization buffer, different start concentrations of LLO for the following refolding process were given. A schematic illustration of the protein refolding set up is depicted in Figure 26. Solubilization of LLO (from 50ml of bacterial suspension) in 5ml solubilization buffer (final concentration of 250µg/ml LLO) and subsequent 1:10 dilution with the different solutions (PBS, PBS with 500mM NaCl, acidic buffer, ddH_2O) showed a strong hemolytic activity of LLO when mixed with pre-washed red blood cells. But after storage at 4°C in the particular buffer solutions,

the activity decreased within 12 hours in all tested buffer systems except when stored at pH = 5, in which the protein is presumed to turn into its active conformation preventing its deactivation by protein aggregation. Following the activity measurements, also the hemolytic activity of LLO stored in the acidic buffer was remarkable decreased, concluding a half-life of approximately 5 days (Figure 27). Simultaneously, a second series of LLO stability experiments was performed with LLO, prediluted in the double amount of solubilization buffer (10ml of 4M Gnd-HCl). The following refolding dilutions, buffers and storage conditions, respectively, were performed equally to the experiment described above. As expected, the protein stability was remarkable increased in all tested buffer systems (Figure 28). A 10-fold increase in protein stability was observed with all buffer systems. Furthermore, like in the above described experiment, highest LLO stability was performed after the storage at pH = 5. Furthermore, a decrease of the concentration of LLO (under 40µg/ml) prior to refolding led to an even more increased stability. It can be concluded, that by decreasing the start concentration of LLO prior to the refolding process (by increasing the volume of solubilization buffer), the refolded proteins have a prolonged stability when stored at 4°C.

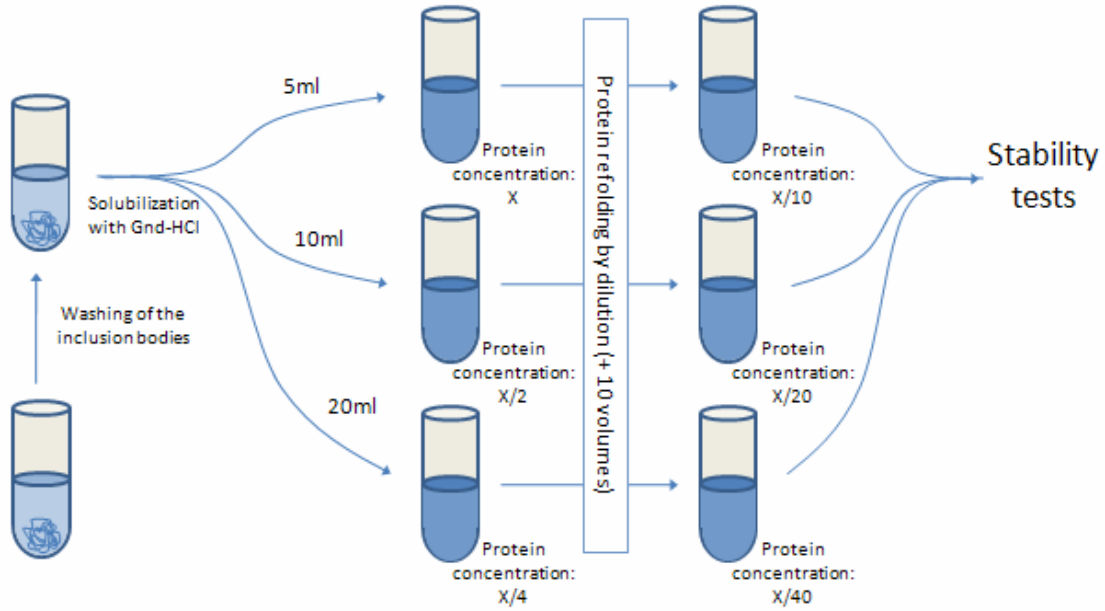


Figure 26: Schematic illustration of the protein refolding set up

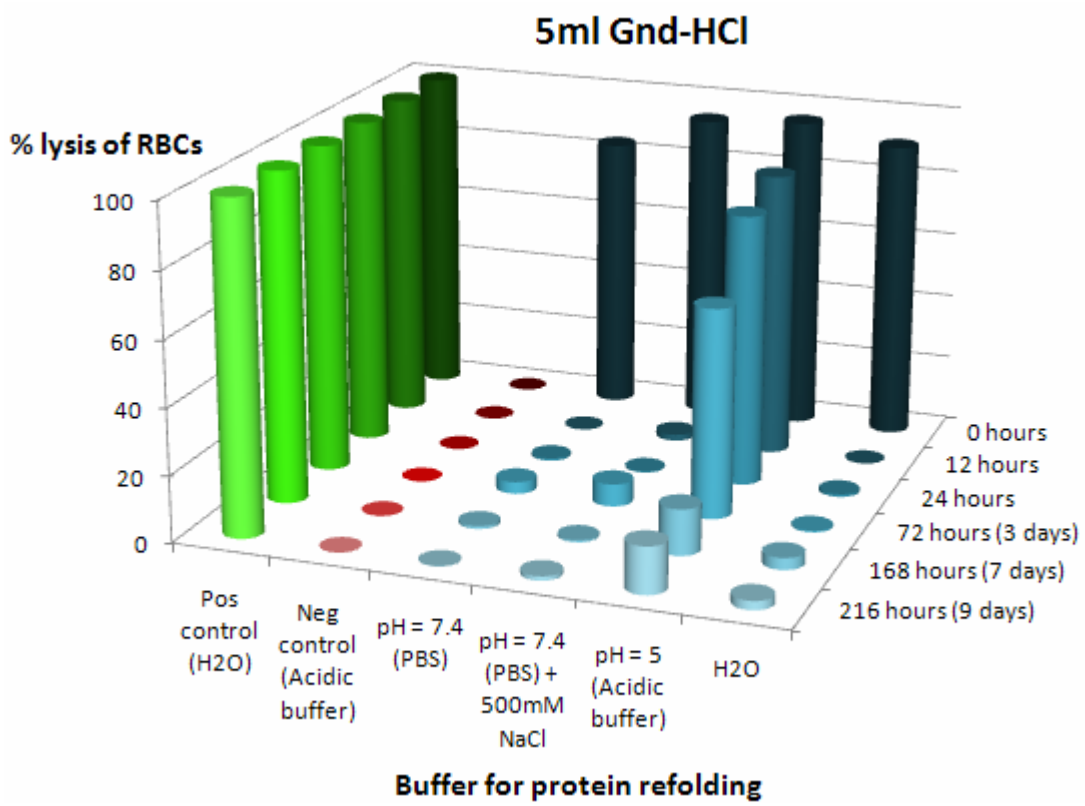


Figure 27: Listeriolysin O (LLO) stability after solubilization of the inclusion bodies in 5ml Gnd-HCl (50ml bacterial suspension) following dilution 1:10 with the depicted buffers. 25ng purified LLO was applied for the lysis of red blood cells (RBCs). For positive control, the resuspension of RBCs in ddH_2O resulted in 100% lysis of RBCs (osmotic lysis). For negative control, RBCs were resuspended in acidic buffer (containing same amounts of Gnd-HCl). For all protein solutions, the storage was performed at 4°C. Measurement of the supernatant after centrifugation was performed photometrically at wavelength of 541nm (percentage relative to 100% lysis of positive control; subtraction of negative control). Protein concentration after 1:10 dilution: 160µg/ml; N = 3

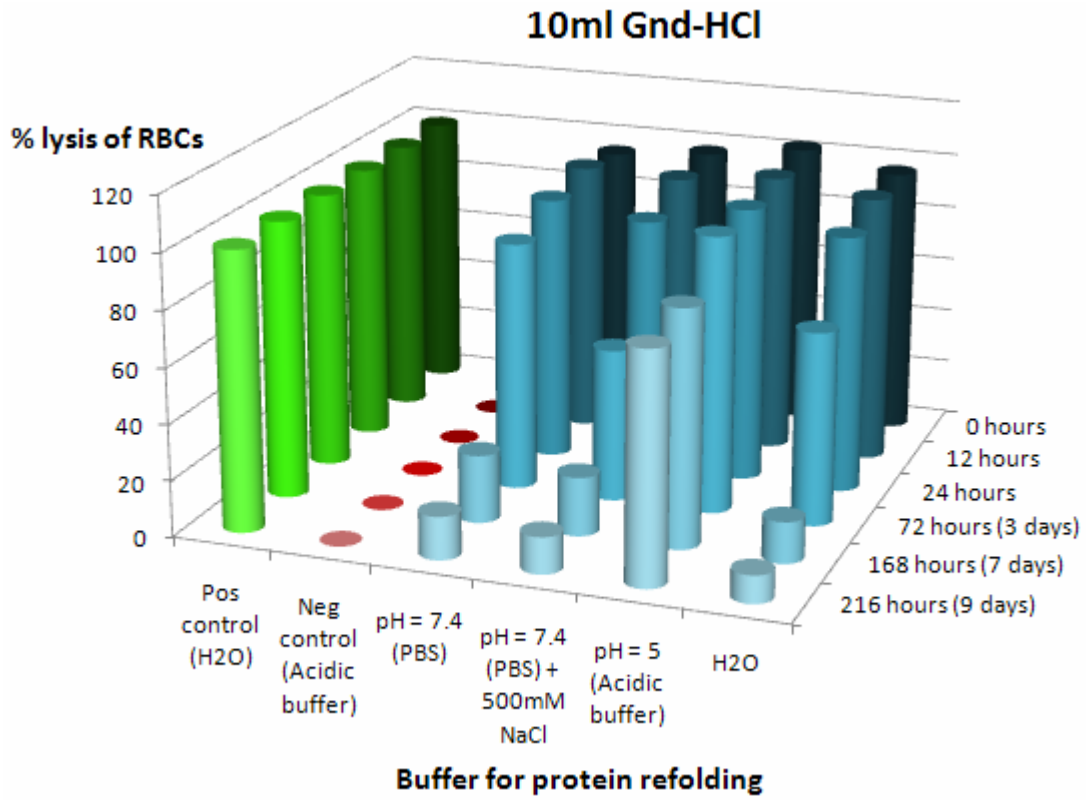


Figure 28: Listeriolysin O (LLO) stability after solubilization of the inclusion bodies in 10ml Gnd-HCl (50ml bacterial suspension) following dilution 1:10 with the depicted buffers. 25ng purified LLO was applied for the lysis of red blood cells (RBCs). For positive control, the resuspension of RBCs in ddH_2O resulted in 100% lysis of RBCs (osmotic lysis). For negative control, RBCs were resuspended in acidic buffer (containing same amounts of Gnd-HCl). For all protein solutions, the storage was performed at 4°C. Measurement of the supernatant after centrifugation was performed photometrically at wavelength of 541nm (percentage relative to 100% lysis of positive control; subtraction of negative control). Protein concentration after 1:10 dilution: 80µg/ml; N = 3

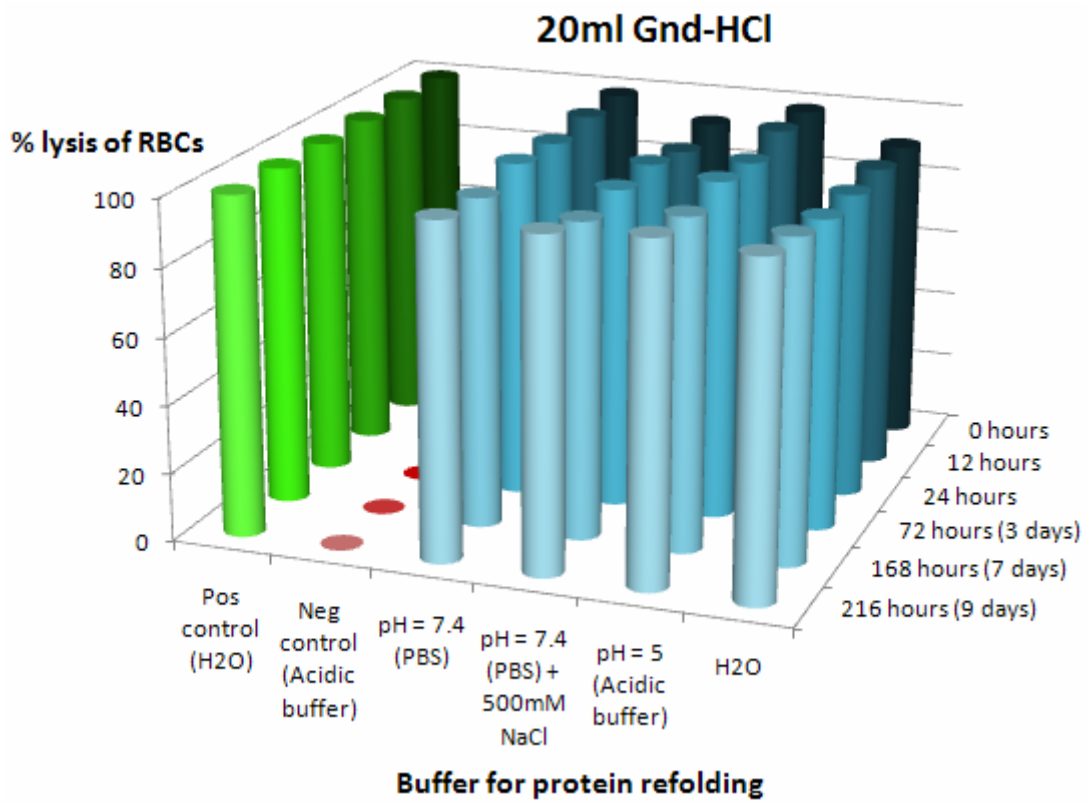


Figure 29: Listeriolysin O (LLO) stability after solubilization of the inclusion bodies in 20ml Gnd-HCl (50ml bacterial suspension) following dilution 1:10 with the depicted buffers. 25ng purified LLO was applied for the lysis of red blood cells (RBCs). For positive control, the resuspension of RBCs in ddH_2O resulted in 100% lysis of RBCs (osmotic lysis). For negative control, RBCs were resuspended in acidic buffer (containing same amounts of Gnd-HCl). For all protein solutions, the storage was performed at 4°C. Measurement of the supernatant after centrifugation was performed photometrically at wavelength of 541nm (percentage relative to 100% lysis of positive control; subtraction of negative control). Protein concentration after 1:10 dilution: 40µg/ml; N = 3

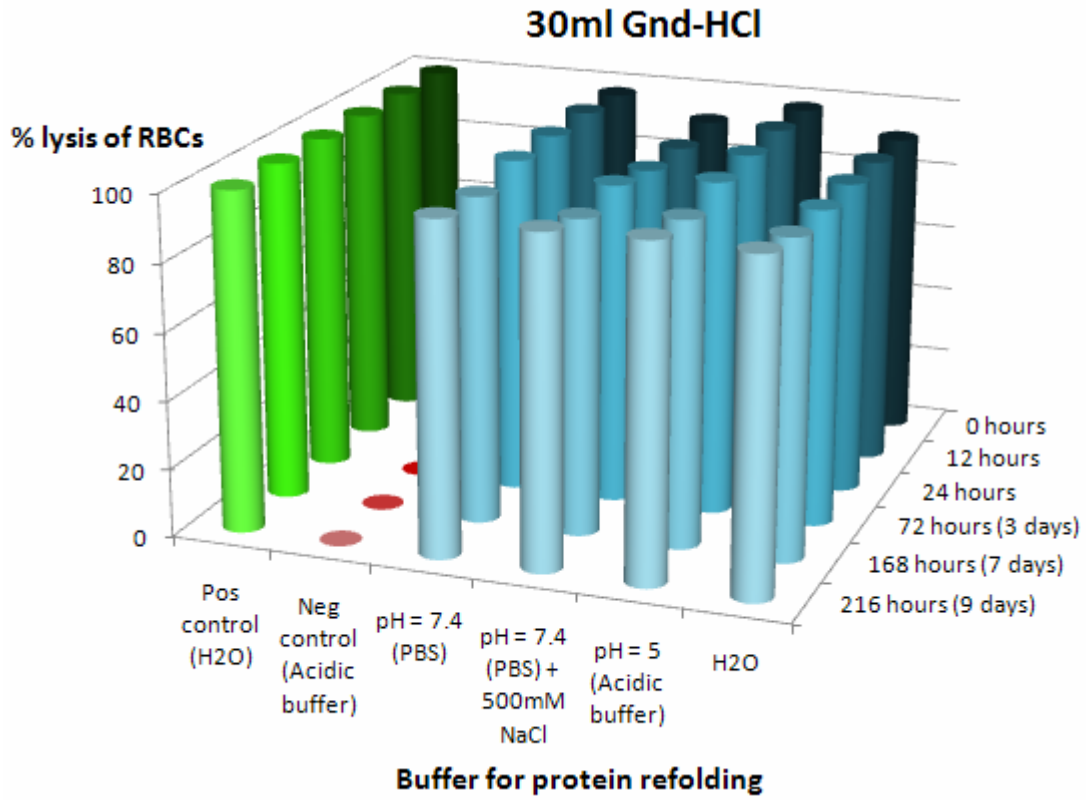


Figure 30: Listeriolysin O (LLO) stability after solubilization of the inclusion bodies in 20ml Gnd-HCl (50ml bacterial suspension) following dilution 1:10 with the depicted buffers. 25ng purified LLO was applied for the lysis of red blood cells (RBCs). For positive control, the resuspension of RBCs in ddH_2O resulted in 100% lysis of RBCs (osmotic lysis). For negative control, RBCs were resuspended in acidic buffer (containing same amounts of Gnd-HCl). For all protein solutions, the storage was performed at 4°C. Measurement of the supernatant after centrifugation was performed photometrically at wavelength of 541nm (percentage relative to 100% lysis of positive control; subtraction of negative control). Protein concentration after 1:10 dilution: 20µg/ml; N = 3

After the observation of LLO stability when stored at 4°C, the next series of experiments was designed to determine whether LLO can be frozen in liquid nitrogen, stored at -80°C, and thawed again without any forfeiture of the protein activity. After solubilization of LLO, the protein samples were diluted 1:10 in either PBS or PBS in the presence of 500mM NaCl and 100µl aliquots were transferred immediately after the refolding process to liquid nitrogen and stored at -80°C. The protein samples were thawed at different time points and tested for their hemolytic activity (same conditions as above). As depicted in Figure 31, the freezing process showed no remarkable loss in the protein activity in the time period of over 36 days (and therefore can be stored over long periods at -80°C).

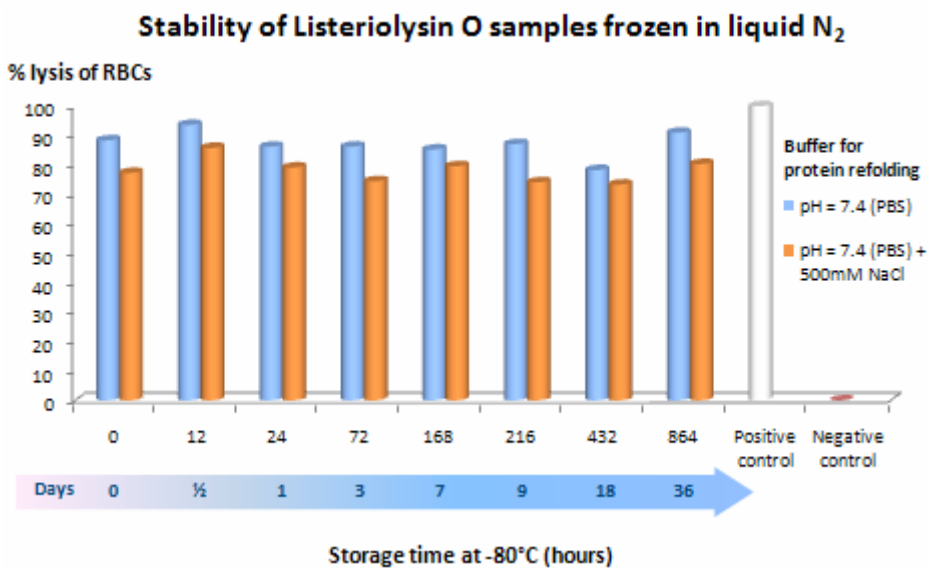
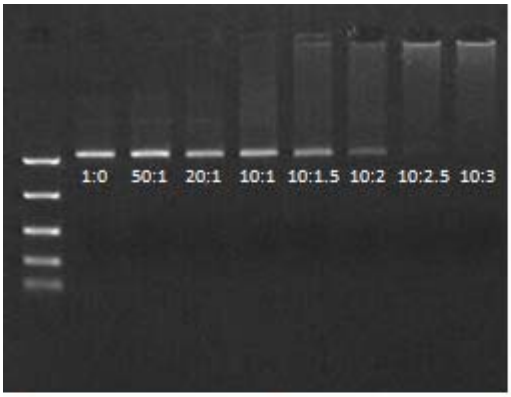


Figure 31: Stability of Listeriolysin O (after refolding step described in Figure 27) frozen in liquid nitrogen, stored at -80°C and thawed after different time points. The results indicate that LLO remains stable during the freezing process and can be stored over long periods at -80°C. The activity of LLO is still active (72 days, end of study), N = 2

3.5. Preliminary DNA complexation studies with poly-L-lysine

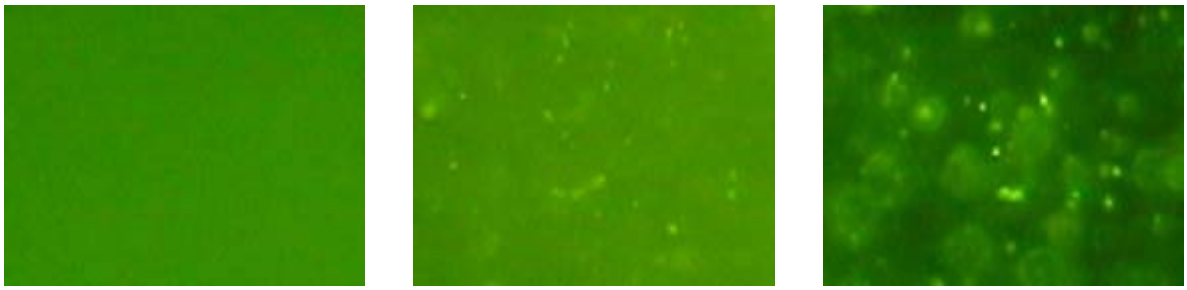
The DNA binding capacity of poly-L-lysine (PLL) with respect to the molar ratio of DNA and PLL was investigated prior to cell transfection analyses using poly-L-lysine as DNA carrier system. Due to the increased size (and decreased negative net surface charge) of the DNA complexed with poly-L-lysine, the migration velocity during electrophoresis in an agarose gel was influenced and therefore the complexation potential of poly-L-lysine with DNA was demonstrated (Figure 32).



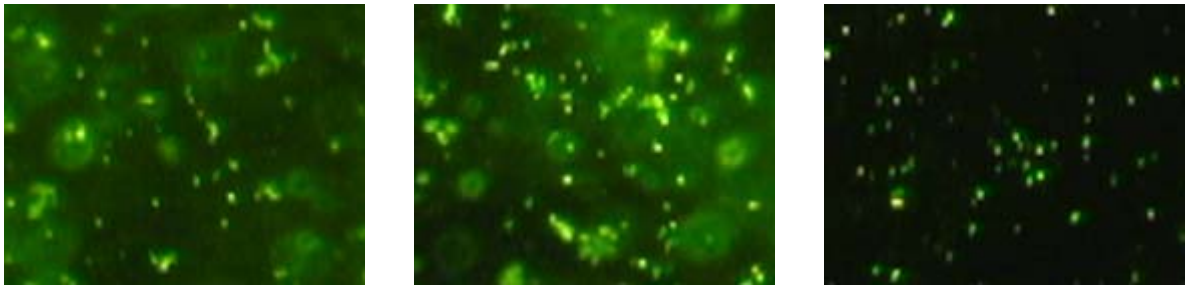
Ratio (µg)	µl (DNA)	µl (PLL)
1:0	2	0
50:1	2	0.5
20:1	2	1
10:1	2	2
10:1,5	2	3
10:2	2	4
10:2,5	2	5
10:3	2	6

Figure 32: Verification of the condensation capacity of poly-L-lysine (PLL) of about 30kDa at different molar ratios of plasmid DNA and PLL.

Furthermore, in order to test the characteristics and the behavior of the applied DNA dye SYBR green under the fluorescence microscope, preliminary DNA condensing studies with poly-L-lysine (PLL, 15,000-30,000Da) were performed prior to the cell transfection studies with the DNA/PLL complexes. Total DNA complexation was observed within 90 seconds following addition of equimolar amounts of PLL (Figure 33).



a) Without PLL b) 10sec c) 30sec



d) 50sec e) 70sec f) 90sec

Figure 33: Condensation of DNA labeled with SYBR green. The DNA condensation was performed with the addition of poly-L-Lysine (15,000-30,000Da) at a molecular ratio of 1 (time points were defined following addition of poly-L-lysine). DNA labeled with SYBR green prior to the addition of poly-L-lysine (PLL) (a), DNA complexes after 10 seconds following addition of PLL (b), DNA complexes after 30 seconds following addition of PLL (c), DNA complexes after 50 seconds following addition of PLL (d), DNA complexes after 70 seconds following addition of PLL (e), DNA complexes after 90 seconds following addition of PLL (f). Magnification = 250x

3.6. Effect of LLO in cell transfection

In order to demonstrate the influence of LLO in the uptake of DNA particles without influencing the transfection system by the addition of proteins, poly-L-lysines (15,000-30,000Da) were used as stable DNA condensing agents. By pre-labeling the DNA with a fluorescence dye (SYBR green), the uptake of the DNA, pre-complexed with PLLs, was observed under the fluorescence microscope within the first 15 minutes following transfer to C2C12 cells in absence and presence of LLO. In the absence of LLO, an entrapment of the particles and the fluorescence dye inside the lysosomes was observed after 15 minutes following transfection (Figure 34), whereas by the addition of LLO prior to transfection, the entrapment was significantly decreased allowing the access into cell nucleus (Figure 35).

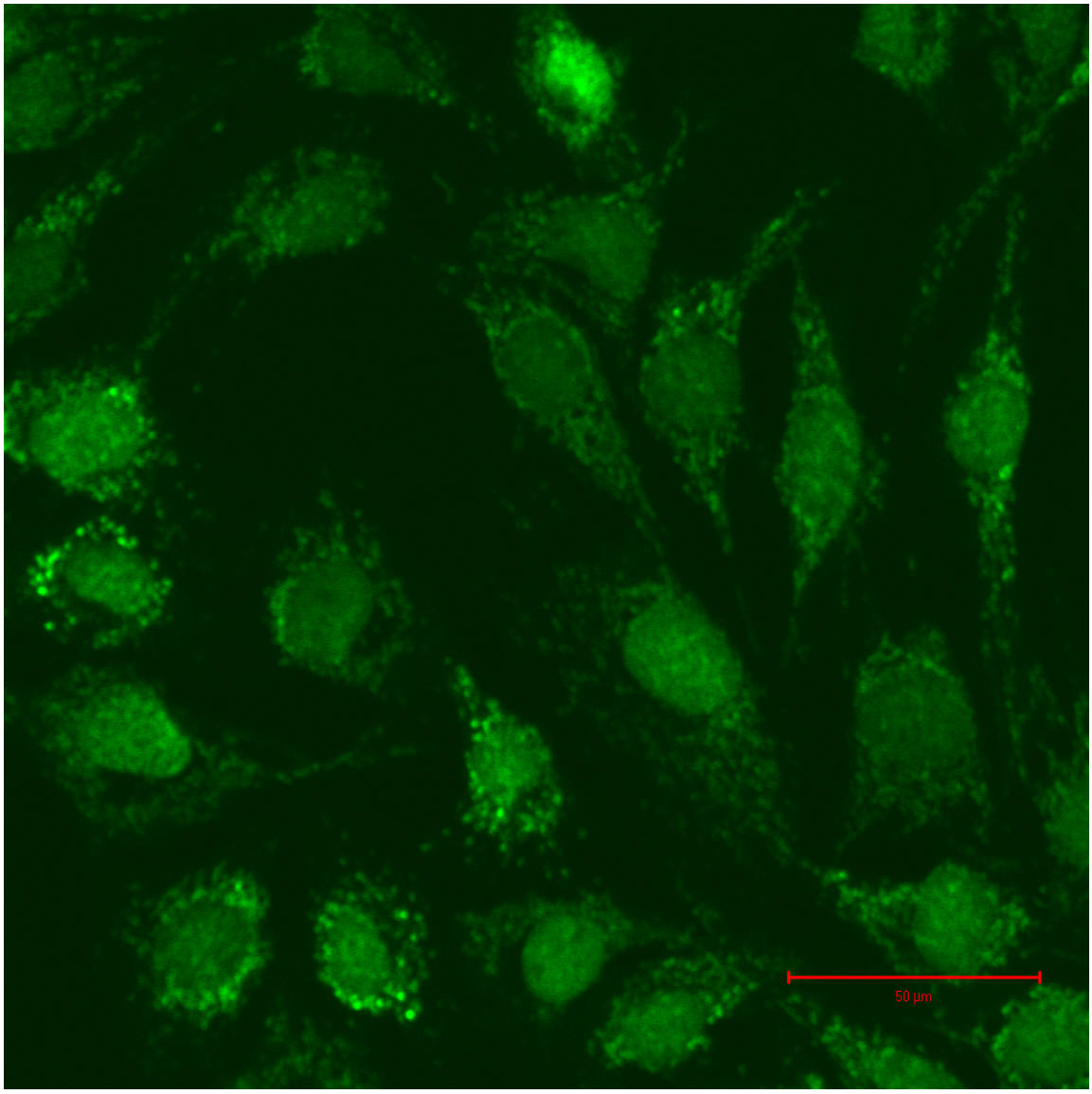


Figure 34: C2C12 cells after the addition of the DNA labeled with SYBR green and complexed with equimolar amounts of poly-L-lysine. Fluorescence microscopy was performed 15 minutes following the addition of the complexes.

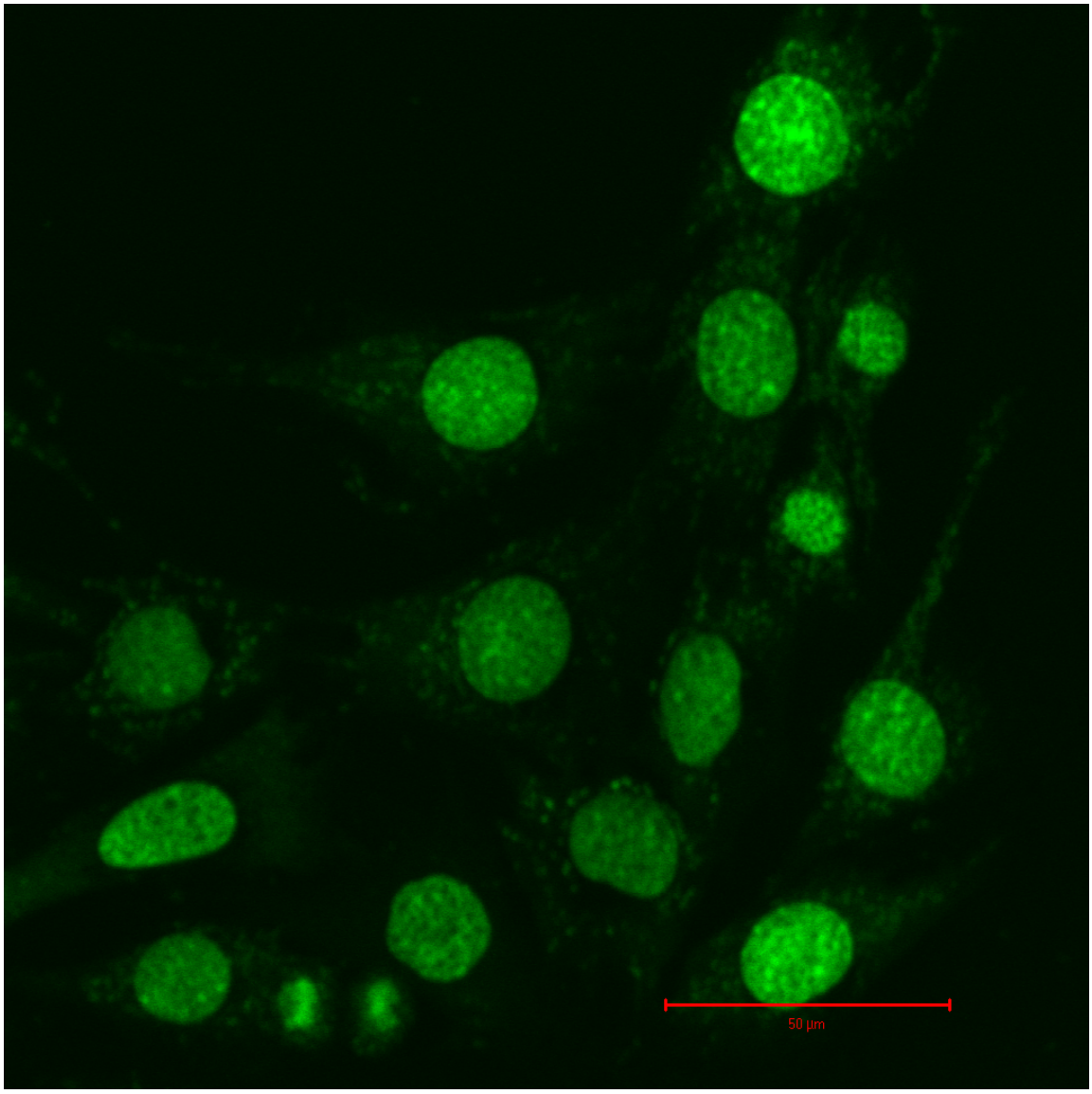


Figure 35: C2C12 cells after the addition of the DNA labeled with SYBR green and complexed with equimolar amounts of poly-L-lysine and 100ng of purified Listeriolysin O. Fluorescence microscopy was performed 15 minutes following the addition of the complexes.

4. Discussion

During the infection process, *Listeria monocytogenes* releases low levels of LLO causing the burst of the lysosomes of target cells to gain access into the cytosol [197, 200]. In order to use the hemolytic toxin as an auxiliary protein to avoid the lysosomal degradation of the introduced DNA, sufficient amounts of pure LLO have to be produced. The results in this work demonstrate that the purified LLO was able to enhance the delivery of macromolecules into the cytosol of mouse skeletal myoblast precursor cells by disrupting the lysosomal compartments. In comparison, enhanced delivery of liposomal content by the presence of LLO [205] as well as the delivery of proteins using *E. coli* as LLO expressing shuttle vector [207] was demonstrated using macrophages as the target cell system. In contrast to the fact that *L. monocytogenes* naturally infects macrophages, this work has demonstrated, that the hemolytic activity of LLO inside the lysosomes of target cells is not cell-type specific and therefore LLO can be used to burst the lysosomes in probably all cell types.

4.1. Expression of LLO in *E. coli* BL21(DE3) and stability assay

The expression of LLO in *E. coli* was performed described in section 2.3.5. In contrast to other published results [312-314], the expressed recombinant protein was assembled in inclusion bodies during the protein expression in *E. coli*. The addition of triton X-100 is commonly used as an additional reagent for washing inclusion bodies. But due to the hemolytic property of triton X-100, washing of the inclusion bodies with triton X-100 was avoided in order not to influence the subsequent hemolytic assay. Additionally, because of the aggregation of LLO after concentration, the subsequent His-tag affinity chromatography was not performed until its maximum loading capacity. Optionally, dialysis was performed prior affinity chromatography in order to remove the leftovers of the solubilization buffer after the refolding by dilution (section 2.3.6.6.).

4.2. LLO stability assay

Once, the lysosomal membrane is disrupted by LLO, the hemolytic protein has to be turned off in order to prevent the subsequent lysis of the eukaryotic cell membrane. Therefore, LLO undergoes a conformational change when exposed to physiological pH conditions leading to intermolecular protein aggregation [201, 202]. This natural property of LLO has to be taken into account during the discovery of ideal storage conditions giving rise to prolonged protein stability. The results support the assumption, that the exposition of LLO to pH = 5 drives the protein into its more stable active conformation, leading to an enhanced life-span. Additionally, the stability of LLO increased when the protein concentration was decreased. Assumable, that by decreasing the concentration of LLO, the force for intermolecular protein aggregation was also decreased.

In contrast, by exposing LLO to physiological pH conditions, the protein was deactivated. The aggregation of LLO is commonly performed when the following physiological conditions (temperature > 33°C, pH > 7) were given. Therefore, by changing only one environmental parameter by decreasing the temperature to 4°C (storage condition), the proteins remained active several days (Figure 28). By further decrease of the protein concentration prior the refolding process, the stability of LLO was even more prolonged (documented for 9 days, verified at least 2 months following the protein refolding, data not shown). The results indicate, that the lower the protein concentration the lower the probability of protein aggregation. Additionally, in contrast to the observations described in [203], no differences in stability in the presence and absence of 500mM NaCl was observed. Contrary, LLO in the presence of 500mM NaCl even showed a slightly lower hemolytic activity.

4.3. Effect of LLO during gene delivery into eukaryotic cells

To test the potential of LLO to enhance the probability of the delivery of complexed particles by bursting the lysosomal compartments, C2C12 cells were transfected with different transfection reagents in the presence and absence of LLO. It was not reproducible demonstrating enhanced cell transfection with fluorescence vectors (pDsRed-Express-C1,

pCDNA3-EYFPHis) in the presence of LLO by using commercially available transfection reagents (Lipofectamine 2000, ExGen, TurboFect), due to the sensitivity of these cationic lipids to other proteins present in the solution. As assumed, the presence of LLO decreased the transfection efficiency of Lipofectamine 2000 (L2K) dramatically, and proportionally to the applied protein concentration. In comparison, LLO replaced by bovine serum albumin (BSA) showed nearly the same effects of hindering the cell transfection (data not shown). Furthermore, due to significant changes in the efficacy of cell transfection even after small alterations in the protein concentration and molecular weight, it was not reproducible to compare the transfection efficiencies in presence of either LLO or BSA, even if the same concentrations were applied. To date, no demonstration of the effect of LLO in enhancing the expression of introduced exogenous DNA due to the higher transfection efficacy has been demonstrated in the literature. Kyung-Dall Lee et al. has shown the release of fluorescence dye when packed in liposomes in the presence of LLO [205], and Higgins et al. has shown similar lysosome disrupting effects of LLO in *in vitro* experiments. Additionally, LLO was used to introduce a toxin for an effective anti-tumor therapy [206].

Furthermore, it has to be mentioned, that SYBR green is a cell permeable fluorescence dye, and therefore an influx of the dye into the cells was observed starting after 30 minutes. Due to this reason, observations above 15 minutes would not lead to any reproducible results, and therefore all observations were performed at most within 10-15 minutes post-transfection. Moreover, the localization of the labeled exogenous DNA was hard to define due to the leftovers of unbound SYBR green, transformed together with the labeled, PLL-complexed DNA. In fact, it is assumable, that this free SYBR green (or SYBR green dissolved from the introduced DNA) was engulfed by the target cells and diffused into the cell nucleus causing fluorescence labeling of the genomic DNA. Anyway, the uptake of the DNA/PLL particles was observed within 15 minutes under light microscopy.

However, in order to test the potency of purified LLO to disrupt the lysosomes, a comparison of the destiny of the fluorescent dye in the presence and absence of LLO was sufficient to conclude the effects of LLO.

4.4. Possible applications

In vivo cell transfection methods are still inadequate due to several limiting factors like inefficient cellular uptake, lysosomal degradation and nuclear import of introduced therapeutic genes. By the adaption of bacterial or viral proteins helping to overcome specific barriers, like the combination of LLO with other transfection methods lacking an efficient mechanism to overcome the lysosmal degradation, the efficacy of the gene delivery could be increased. In contrast to other synthetic or naturally derived hemolytic compounds, LLO has the advantage of its pH dependent hemolytic activity displayed only inside the lysosomes and its low cytotoxicity compared to other hemolytic proteins [190, 315]. Furthermore, LLO is degraded when located inside the cytosol, therefore featuring an additional protection of the cell membrane of the host cell [316], which makes the protein to an ideal candidate to increase the efficacy of gene delivery applications.

5. Summary

In contrast to strategies based on the introduction of transgenic cells expressing growth factors (*ex vivo* therapy), or the direct administration of recombinant growth factors into target systems, *in vivo* gene therapy approaches (introduction of therapeutic plasmids encoded for growth factors) provide a promising alternative associated with lower manufacturing costs, higher safety and increased bioactivity of the produced proteins (due to host-specific post-translational modifications and correct folding of the locally produced growth factors). Utilizing viral particles for high transfection efficiencies (high efficiency for DNA introduction into target cells *in vivo*) is unsuitable due to the high immunogenicity and the nature of some viral vectors to manipulate the host genome.

On the other hand, non-viral gene delivery methods provide high safety and low immune response, but are limited in their transfection efficiency. Main reasons for impaired transfection capacity are confined cellular uptake of the exogenous DNA, the lysosomal degradation after uptake and the low efficiency of the introduced plasmid DNA to diffuse into the cell nucleus.

This study was focused on the lysosomal degradation which highly limits the transfection efficiency. In order to avoid the lysosomal degradation of introduced therapeutic DNA and therefore to significantly increase the probability of the engulfed DNA to overcome the lysosomal barrier, the influence of the hemolytic protein listeriolysin O, derived from the intracellular bacteria *Listeria monocytogenes*, was tested in gene delivery approaches. During the life cycle of *Listeria monocytogenes*, the hemolytic protein is secreted after the engulfment of the bacteria in order to disrupt the lysosomes and to ensure the bacterial entry into the cytosol of target cells. The main advantage of listeriolysin O is its hemolytic activity to disrupt eukaryotic membranes restricted only under acidic conditions (below pH = 6) as given in the lysosomes. Due to this pH dependent activity of listeriolysin O, and the subsequent deactivation by aggregation after exposition to physiological pH conditions (pH = 7), the subsequent disruption of the host cell membrane is avoided preventing the host cell from lysis. Based on this, listeriolysin O is a promising auxiliary protein for the enhancement of the transfection efficiency.

The *hlyA* gene, encoded for Listeriolysin O and lacking its secretion signal, was C-terminally linked to a polyhistidine tag and cloned into a bacterial expression vector following expression in *E. coli*. Subsequent optimization of the protein purification was performed to attain high yields of pure and bioactive LLO, extensively exceeding presently published results concerning total yield of LLO and effort of the purification method. The influence of the purified LLO in cell transfection approaches was tested *in vitro*. The DNA was prelabeled with the fluorescence dye and complexed with the polycationic DNA carrier poly-L-lysine (PLL). Particles were transferred to the cells and the uptake efficiency of the particles was observed in the presence and absence of LLO after 15minutes under fluorescence excitation to confirm the subsequent access of the DNA dye into the cell nucleus after lysosomal disruption by LLO.

By comparing the uptake of the fluorescence labeled DNA microparticles, a remarkable increase of the uptake was observed within the first 15 minutes under the fluorescence microscope when LLO and the DNA microparticles were simultaneously transferred to the cells. In contrast, transfection of cells in the absence of LLO showed an entrapment of the microparticles inside the lysosomes within the same time period.

Zusammenfassung

Im Gegensatz zu Strategien basierend auf die Einführung von transgenenen Zellen, die Wachstumsfaktoren exprimieren, oder die direkte Administration von rekombinanten Wachstumsfaktoren *in vivo*, bietet die Gentherapie (Einführung von exogener DNA kodierend für einen Wachstumsfaktor) eine einfach anzuwendende, kostengünstige Alternative, verbunden mit erhöhter Bioaktivität der exprimierten Wachstumsfaktoren (durch wirtszell-spezifischer post-translationaler Modifikation und korrekter Faltung der lokal durch die Zielzellen produzierten Wachstumsfaktoren).

Jedoch ist der Einsatz von viralen Vektoren, die eine effiziente Einschleusung von Fremd-DNA in Zielzellen *in vivo* garantieren, wegen der Auslösung einer Immunantwort und das Integrieren der Fremd-DNA in das Genom der Zielzelle, ungeeignet.

Nicht-virale Vektoren hingegen bieten eine hohe Sicherheit und erzeugen eine geringfügige Immunantwort, sind jedoch in ihrer Fähigkeit Zielzellen *in vivo* zu transfizieren, beschränkt. Die physische und chemische Barriere der Zellmembran, der lysosomale Abbau und die

geringe Effizienz der Diffusion der eingeführten DNA in den Zellkern limitiert die Wirksamkeit der Transfektion erheblich.

Diese Arbeit war ausgerichtet, den lysosomalen Abbau eingeführter Makromoleküle wie DNA zu verhindern um die Transfektionseffizienz zu erhöhen. Um den lysosomalen Abbau zu vermeiden und dadurch die Wahrscheinlichkeit der Einführung therapeutischer DNA in das Zytosol bzw. den Zellkern zu erhöhen, wurde versucht, mithilfe eines bakteriellen Proteins (Listeriolysin O) die Lysosomen während der endozytischen Aufnahme der DNA zu zerstören. Durch die pH-abhängige Aktivierung der Hämolysins in der lysosomalen Umgebung werden die Lysosomen zerstört und die endozytotisch aufgenommene therapeutische DNA ins Zytosol freigesetzt.

Listeriolysin O wurde in E. coli exprimiert und anschließend biochemisch aufgereinigt, um dessen Einfluss auf die DNA-Transfektion in eukaryotischen Zellen zu ermitteln. Bei Anwesenheit von Listeriolysin O zeigte sich eine erhöhte Präsenz von aufgenommenen Molekülen (DNA, Farbstoff) im Zytosol der Zielzellen.

6. References

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7. Appendix

CATATGAAGGATGCATCTGCATTCAATAAAGAAAATTCAATTTCATCCATGGCACCACCAGCATCTCCGCCTGCAAGTCCTA
AGACGCCAATCGAAAAGAAACACGCGGATGAAATCGATAAGTATATACAAGGATTGGATTACAATAAAAACAATGTATTA
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ACGTTGCTCAATTCAACATTTCTTGGGATGAAGTAAATTATGATCCTGAAGGTAACGAAATTGTTCAACATAAAAACTGGA
GCGAAAACAATAAAAGCAAGCTAGCTCATTTCACATCGTCCATCTATTTGCCTGGTAACGCGAGAAATATTAATGTTTACG
CTAAAGAATGCACTGGTTTAGCTTGGGAATGGTGGAGAACGGTAATTGATGACCGGAACTTACCACTTGTGAAAAATAGA
AATATCTCCATCTGGGGCACCACGCTTTATCCGAAATATAGTAATAAAGTAGATAATCCAATCGA*ACATCATCATCATCATC*
*AT***TAA**GGATCC

Table 14: Nucleotide sequence of LLO lacking its secretion signal verified by DNA sequencing. C-terminal His-Tag (italic), flanked restriction sites NdeI and BamHI (underlined).

Curriculum vitae

Personal details	**Name**	Ara Hacobian
	Address	Wacholderweg 17a 1210, Vienna
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Education	**1988-1992**	Elementary School in Vienna
	1992-2000	College Preparatory High School BRG Ödenburg, Vienna
	2001 to date	Studies in Molecular Biology at the University of Vienna, Campus Vienna BioCenter
	2006	First Degree (equal to Bachelor) in Molecular Biology at the University of Vienna
Military service	**2000-2001**	in Wiener Neustadt
Work experience	**May 2007 - to date**	Master thesis at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (Enhancement of non-viral cell transfection)
	May-June 2007	Campus Vienna Biocenter (Max F.Perutz Laboratories) (RNases in microorganisms)
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	Mai-August 2005	Brain Research Center, Vienna (RNA uptake and transport in neuronal cells)
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